University of Strathclyde
Strathclyde Institute of Pharmacy and Biomedical Sciences

Biochemical and immunological characterisation of *Toxoplasma gondii* macrophage migration inhibitory factor

By
Caroline Sommerville

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has lead to the award of a degree.

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Abstract

Macrophage migration inhibitory factor (MIF) is a key pro-inflammatory cytokine involved in both innate and adaptive immune responses. Specifically, MIF has been implicated in being involved in numerous biological processes including p53 dependent apoptosis and T cell activation. MIF also exhibits two enzymatic properties: tautomerase and oxidoreductase activities through which MIF biological function is suspected to be mediated. Throughout the past 10 years, MIF homologues have been identified in numerous parasitic species including *Brugia*, *Leishmania*, *Trichinella* and *Plasmodium*. Indeed, identification of such homologues has led to the suggestion of immunoregulatory roles for parasite MIFs. A single copy of the MIF gene is found in the genome of the Apicomplexan parasite, *Toxoplasma gondii*. Herein is documented the first report regarding characterisation of *Toxoplasma gondii* macrophage migration inhibitory factor (TgMIF). TgMIF has 25% sequence identity with human MIF and is expressed in both the tachyzoite and bradyzoite life cycle stages. Despite lacking an N-terminal leader sequence for classical secretion, TgMIF is a secreted protein which has a predicted molecular weight of 37.5kDa in its trimeric form. TgMIF was cloned and recombinant C-terminal His tagged protein was produced and used in biochemical assays to determine TgMIF catalytic activity. TgMIF has a measurable tautomerase activity of 1476 μmoles/min/mg which is 81 and 91 fold less than that of human and mouse MIF respectively, yet comparable to other parasite MIFs. In contrast to human MIF, TgMIF tautomerase activity was not affected by the MIF inhibitor ISO-1. Oxidoreductase activity is minimal, an observation that is consistent with the absence of a conserved cysteine residue that has been shown in other homologues to be necessary for optimum activity. The crystal structure of TgMIF has been determined at 1.82Å resolution and has a trimeric ring architecture similar to mammalian MIF. Indeed, TgMIF structural analysis highlighted substitutions in and around the tautomerase active site that would account for the reduced tautomerase activity and the inability of ISO-1 to inhibit this activity. Recombinant endotoxin free TgMIF is capable of driving IL-8 production from human peripheral blood mononuclear cells. Furthermore, TgMIF is capable of inducing ERK-MAPK activation in murine bone marrow-derived macrophages whilst having no significant effect on the expression of receptor molecules, CD74 and CD44. Finally, stimulation of bone marrow-derived macrophages with TgMIF and LPS,

results in an apparent down regulation of TLR4 mRNA levels. A role for TgMIF in modulation of host immune responses, in order to create a favourable environment for parasite survival is therefore suggested.

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Abbreviations

3-BP	3-bromopyruvate
4-IPP	4-iodo-6-phenylpyrimidine
4-OT	4-oxalocrotonate tautomerase
ACTH	Adrenocorticotropic
AIDS	Acquired immune deficiency syndrome
AMA	Apical membrane antigen
AP-1	Activator protein 1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAG-1	Bradyzoite antigen 1
BCA	Bicinchoninic acid
BmMIF	*Brugia malayi* MIF
CCR	Chemokine receptor
CD	Cluster of differentiation
cDNA	complementary DNA
CHMI	5-carboxymethyl-2-hydroxymuconate isomerase
CIA	Collagen induced arthritis
CMI	Cell mediated immunity
CNS	Central nervous system
COP	Coat protein complex
COX	Cyclooxygenase
cPLA2	cytosolic phospholipase A2
DC	Dendritic cell
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK MAPK	Extracellular regulated kinase/ mitogen - activated protein kinase

ESAg	Excretory secretory antigens
EST	Expressed sequence tag
EtMIF	*Eimeria tenella* MIF
FCS	Foetal calf serum
GC	Glucocorticoid
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
HED	2-hydroxyethyldisulphide
HEPES	(4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid)
HFF	Human foreskin fibroblast
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HsMIF	*Homo sapiens* MIF
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified dulbecco's medium
iNOS	inducible nitric oxide synthase
IPTG	Isopropyl β-D-1- thiogalactopyranoside
ISO-1	(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid
Jab1	Jun activating binding protein 1
kDa	kilo Daltons
LAL	Limulus ameobocyte lysate
LBP	LPS binding protein
L.DOPA	L-dihydroxyphenylalanine
LmMIF	*Leishmania major* MIF
LPS	Lipopolysaccharide
MΦ	Macrophage
MCP	Monocyte chemotactic protein

MHC	Major histocompatibility complex
Mic	Microneme
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
MJ	Moving junction
MKP	MAPK phosphatase
mRNA	messenger RNA
MyD88	Myeloid differentiation primary response gene 88
MW	Molecular weight
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NK	Natural killer
NO	Nitric oxide
NTA	Nitroacetic acid
OT	Ocular toxoplasmosis
PBMC	Peripheral blood mononuclear cells
PbMIF	*Plasmodium berghei* MIF
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PfMIF	*Plasmodium falciparum* MIF
PGE2	Prostaglandin E2
PV	Parasitophorous vacuole
RA	Rheumatoid arthritis
RFLP	Restriction fragment length polymorphisms
RMSD	Root mean square deviation
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROP	Rhoptry
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
SAG-2a	Surface antigen 2a
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.	Standard error

SNP	Sodium nitroprusside
SO	Super oxide
STAg	Soluble *Toxoplasma* antigen
STAT	Signal transducer and activator of transcription
TBP	TATA box binding protein
TE	Toxoplasmic encephalitis
TFB	Transformation buffer
TgCyp18	*T. gondii* cyclophilin18
TGF	Tumor growth factor
TgHSP70	*T. gondii* heat shock protein 70
TgMIF	*Toxoplasma gondii* MIF
TgPRF	*T. gondii* profilin
Th	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	T regulatory
TsMIF	*Trichinella spiralis* MIF
TSS	Toxic shock syndrome
TSST	Toxic shock syndrome toxin
Trx	Thioredoxin
VCAM	Vascular cell adhesion molecule
X-gal	5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside

Chapter 1

Introduction

1.0 Discovery of *Toxoplasma gondii*

Toxoplasma gondii was discovered in 1908 by Nicolle and Manceaux who initially mistook this newly identified parasite for *Leishmania*. *T. gondii* was named so due to its classical arc like shape (toxo = arc or bow) and its discovery within the tissue of the small rodent gundi, *Ctenodactylus gundi*. Almost simultaneously, Splendore (1908) also isolated *T. gondii* from the rabbit, although he did not go on to name this species. Since then, *T. gondii* has been described as a ubiquitous, obligate intracellular protozoan parasite of the subphyla Apicomplexa. The definitive hosts, not identified until 1969 by Hutchison (1965), are members of the family Felidae. However, *T. gondii* has the capability of infecting almost any warm blooded animal resulting in a huge reservoir of intermediate hosts including mammals and birds. Infection in humans is relatively common worldwide and it has been estimated that one third of the world's population is chronically infected (Dubey, 1998a).

1.1 Epidemiology

T. gondii has developed into three prominent clonal lineages as discovered by studies involving multilocus restriction fragment length polymorphisms (RFLP) at 6 different loci in 106 *T. gondii* isolates (Howe and Sibley, 1995). These lineages are described as type I, type II and type III (Howe and Sibley, 1995; Khan *et al.*, 2005). Amongst the lineages, only two alleles exist at each locus and consequently they differ genetically by less than 1% (Su *et al.*, 2003). Subsequently, it has been suggested that at the origins of *T. gondii* evolution two gene pools produced the recombinant progeny from which only a few strains have since become dominant worldwide.

Type I strains including RH, are the most virulent strains of *T. gondii* and are associated with severe disease and have been shown to kill laboratory mice within 16 days (Saeij *et al.*, 2005). Barragan and Sibley, (2002) demonstrated *in vitro*, that type I strains were significantly better at migrating across biological barriers in comparison to type II and III strains. The ability of the parasite to cross biological barriers such as the gut epithelia and placenta is characteristic of a virulent strain and subsequently type I strains have been linked with congenital toxoplasmosis (Fuentes *et al.*, 2001). Switaj *et al.*, (2006) also suggested a role for type I strains in the development of ocular toxoplasmosis (OT) in immunocompromised individuals. This is consistent with a study in the USA where most immunocompetent individuals suffering from

severe atypical OT were also found to be infected with type I organisms. However, this is in contrast to the United Kingdom, where such forms of OT in immunocompetent individuals are normally due to an infection with a type II strain similar to the ME49 laboratory strain and its derivatives (Saeij *et al.,* 2005). The findings of both of these studies are contrary to those reported by Grigg *et al.,* (2001), who found that type I or recombinant type I-III strains are responsible for OT in healthy individuals in USA.

In France, type II strains have been linked with congenital toxoplasmosis (Ajzenberg *et al.,* 2002). However, this is in contrast to Spain where type I strains have been linked with congenital infection (Fuentes *et al.,* 2001). Therefore, it is difficult to conclusively link a disease phenotype with a specific strain due to geographical discrepancies. Despite type II strains being associated with less severe infection, this can often vary dependent on experimental animal models and route of infection. For example, C57BL/6 mice are susceptible to oral infection with ME49 but resistant to infection via intraperitoneal injection (McLeod *et al.,* 1989). Finally, several studies have shown that type III strains do not influence any particular disease manifestation or outcome (Ajzenberg *et al.,* 2002, Costa *et al.,* 1997, Howe and Sibley, 1995, Boothroyd and Grigg, 2002).

Most of the literature to date states the existence of only three clonal lineages. However, some authors propose the existence of a further two types: type IV and V. This is a somewhat controversial and contradictory area of research at present, however, it is proposed that types IV and V may in fact be recombinant strains of *T. gondii* (Holland, 2004). Recombinant strains are formed by atypical allele combinations and are normally exotic and found in African and South American patients, who appear to have unusual disease manifestations (Saeij *et al.,* 2005).

1.2 LIFE CYCLE

1.2.1 Enteroepithelial

The life cycle of *T. gondii* can be broken down into two stages: enteroepithelial and extraintestinal (Figure 1.1 and Figure 1.2). The enteroepithelial stage is initiated in the definitive host, when either an oocyst or tissue cyst is ingested. Of note, research has shown that a bradyzoite induced cycle is more efficient than an oocyst induced cycle, as nearly all cats secrete infective oocysts 3 to 10 days following bradyzoite ingestion. This is in comparison to ingestion of oocysts or tachyzoites which result in ≤30% of cats secreting oocysts (Dubey and Frenkel, 1976). Following ingestion, oocysts or tissue cysts are digested by enzymes in the stomach and small intestine with younger cysts being more susceptible to digestion by gastric juice (Dubey *et al.*, 1997). Sporozoites or bradyzoites are then released from the oocyst or tissue cyst respectively, where they actively enter the epithelial cells of the intestine (Dubey, 1998a). Here they undergo developments over a 15 day period involving the formation of schizonts from which merozoites are subsequently produced. From these newly formed merozoites, both microgametes and macrogametes are formed in the ileum by gametogenesis. Subsequently, the macrogamete is fertilised by the microgamete which results in the formation of a tough cell wall. The fertilised gamete then develops into a zygote before it transforms into an oocyst. The oocyst is finally released from the wall of the intestine and is passed out in the faeces of the cat. An infected cat can shed *T. gondii* in its faeces for a few weeks following infection, although in this short time a large number of oocysts have been shown to be excreted. These oocysts can then survive in moist soil for 18 months whilst remaining infectious (Frenkel *et al.*, 1975). Additional studies have also demonstrated that oocysts remain infective in water over a range of temperatures from -10°C to 25°C for at least 106 days but up to 200 days (Dubey, 1998b).

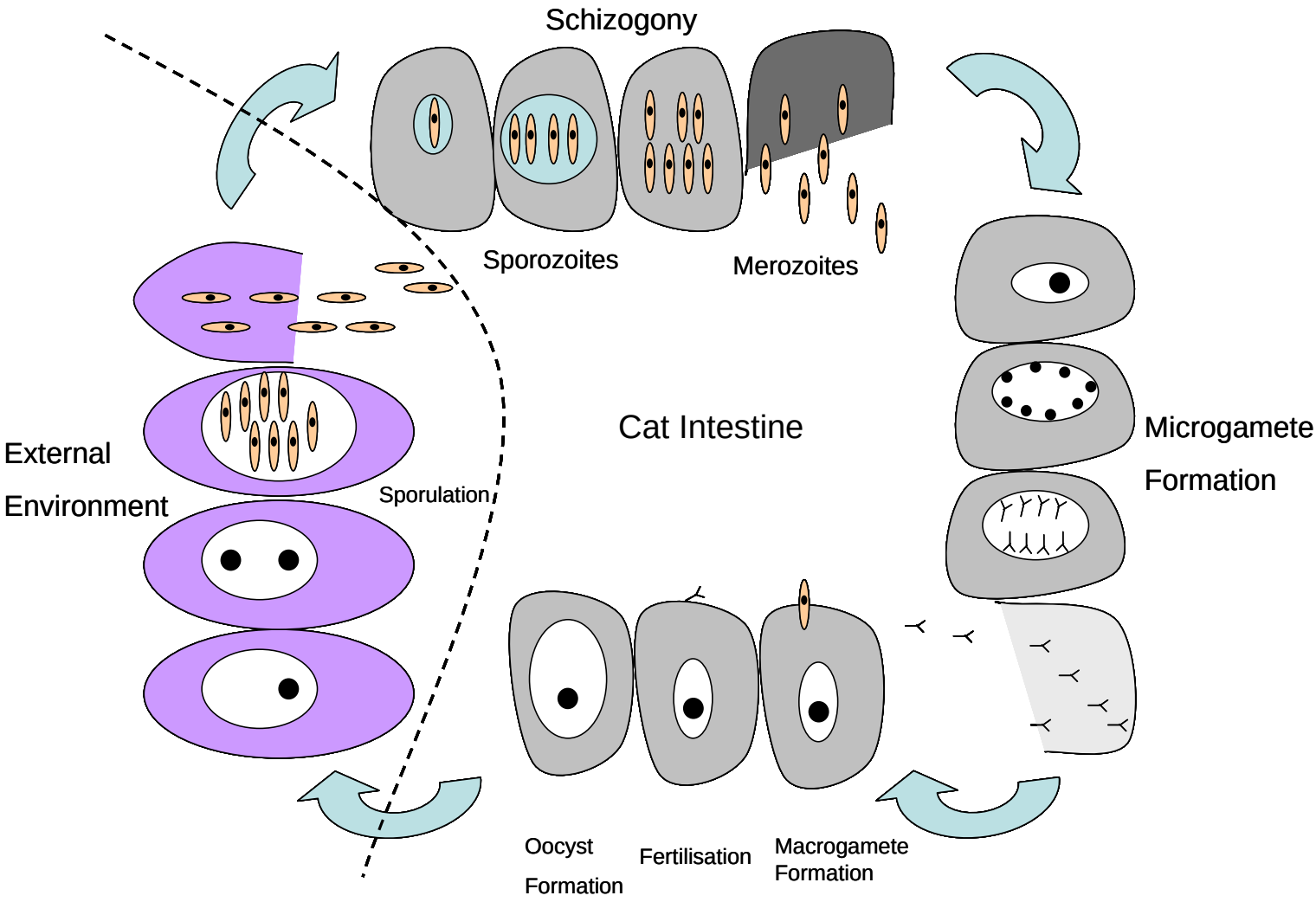


Figure 1.1: The enteroepithelial cycle of *T. gondii*

Following ingestion of oocysts or tissue cysts, sporozoites are released which undergo schizogony to form merozoites that can differentiate into microgametes and macrogametes by gametogenesis. Fertilisation of the macrogamete by the microgamete leads to formation of oocysts in the gut which are then expelled in the faeces of the cat.

1.2.2 The extra-intestinal stage

The extra-intestinal stage of the life cycle, also known as the asexual cycle, is initiated in an intermediate host following ingestion of an oocyst or tissue cyst. Sporozoites are released from the ingested oocyst/tissue cyst and subsequently circulate throughout the body via the bloodstream to various tissues such as the brain. Within the colonised tissues, new host cells are invaded including macrophages and dendritic cells (Aliberti and Bafica, 2005; Kravetz and Federman, 2005) where the parasite undergoes endodyogeny to form tachyzoites, which are representative of the fast dividing stage of infection. Tachyzoites are formed in the parasitophorous vacuole (PV) as soon as 12 to 18 hours post infection and they are subsequently released resulting in infection of neighbouring cells where the endodyogeny process continues (Dubey, 1998a). Following this, tachyzoites undergo transformation into bradyzoites, ultimately resulting in cyst formation (Black and Boothroyd, 2000; Kravetz and Federman, 2005).

The interconversion process of tachyzoites to bradyzoites is important for the establishment of a chronic infection and has been shown to be activated in stress induced environments e.g. extreme pH (Soete *et al.,* 1993). The process is also dependent on heat shock proteins (HSP) as inhibition of HSP production, results in a decrease in bradyzoite formation (Weiss *et al.,* 1998). The interconversion process can be monitored by specific markers such as bradyzoite antigen 1 (BAG1) and also transcripts for enzymes such as lactate dehydrogenases (Yang *et al.,* 1997) and P-type ATPase (Holpert *et al.,* 2001) which have been described at higher levels in bradyzoites than tachyzoites. In addition, there are families of surface antigens (SAG) that are differentially expressed in tachyzoites and bradyzoites (Reviewed Lyons *et al,* 2002). Studies have also suggested that the interconversion process may require IFN-γ and TNF-α (Lyons *et al.,* 2002). Furthermore, most recently, recombinant *T. gondii* cyclophilin-18 (TgCyp18) has been shown to induce production of NO, IL-12, IL-6, IFN-γ and TNF-α via binding to cysteine-cysteine chemokine receptor 5 (CCR). This corresponded with a decrease in parasite proliferation and an increase in bradyzoite formation (Ibrahim *et al.,* 2009). However, recent studies have reported spontaneous conversion of tachyzoites to

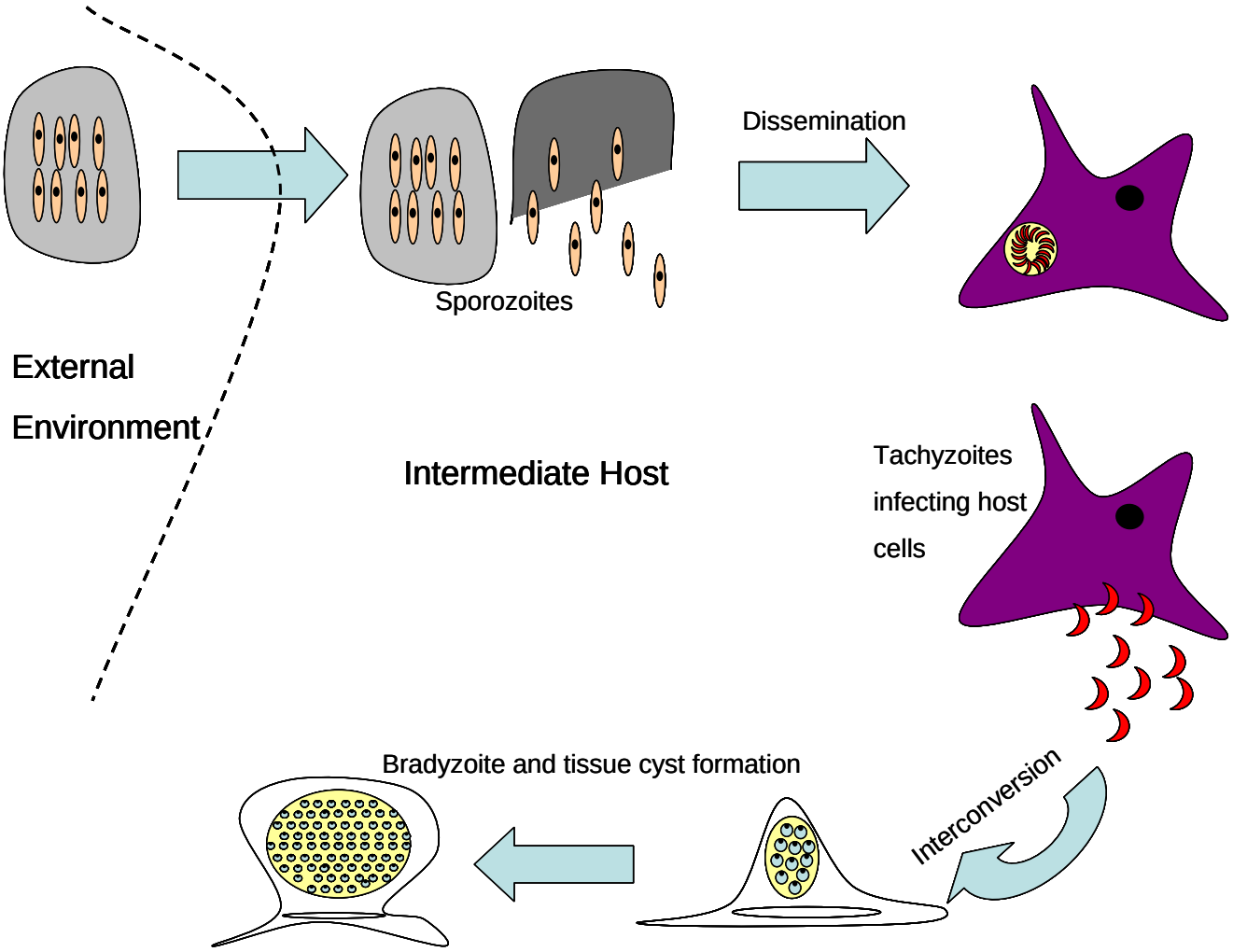


Figure 1.2 The extra intestinal stage of the *T. gondii* life cycle

The extra intestinal stage of the *T. gondii* life cycle occurs in an intermediate host upon ingestion of oocysts from which sporozoites are released. Sporozoites then convert into tachyzoites which disperse throughout the host, infecting new cells. In host cells, tachyzoites undergo replication before egressing from the cell. Tachyzoites are then able to convert into bradyzoites which are representative of the slow dividing stage of infection and are associated with tissue cyst formation.

bradyzoites *in vitro* in skeletal muscle cells (Reviewed Ferreira da Silva *et al.*, 2009) i.e. in the absence of stress triggering mediators. This has prompted suggestion that host cell type is an important factor in cases of spontaneous conversion.

Upon conversion of tachyzoites to bradyzoites, tissue cysts are formed. Tissue cysts are predominantly found in the neural and muscular tissues of the CNS, eye, skeletal and cardiac muscles (Dubey and Frenkel, 1976, Reviewed Tenter *et al.*, 2000). Cyst formation allows the parasite to exist within the host for many years, probably life long, in a state whereby the host remains infected, but is without overt illness (Aliberti and Bafica, 2005). Despite being essential for chronic infection, cyst formation occurs 2-3 days following parenteral infection with tachyzoites or 5-6 days following infection with bradyzoites (Dubey and Frenkel, 1976, Dubey 1997). However it is believed that the periodic rupture of tissue cysts facilitates the release of bradyzoites which are converted to tachyzoites that can reinvade new host cells and ultimately convert back into bradyzoites. This process not only reinforces an existing immune response, maintaining protective immunity, but it also maintains persistence of cysts throughout the hosts lifetime.

Finally, ingestion of tissue cysts results in the release of bradyzoites which can infect the intestinal epithelium of the next host. This initiates the asexual cycle in the intermediate host or the sexual cycle in felids.

1.3 TRANSMISSION

In order to maintain the life cycle, *T. gondii* must be successfully transmitted from definitive to intermediate host, intermediate to definitive host and finally between both definitive and intermediate hosts (Figure 1.3).

A common source of *T. gondii* infection is via the consumption of undercooked meats (Weinman and Chandler, 1954). Animals bred for consumption may become infected from an environmental source and subsequently infection can be passed from cattle to humans if the meat source is undercooked prior to consumption (Afonso, Thulliez and Gilot-Fromont, 2006). In the U.S.A. there is an estimated 225,000 reported cases of toxoplasmosis every year, resulting ultimately in 750 deaths. Consequently, infection with *T. gondii* is the third most common fatal food borne illness (Mead *et al.*, 1999).

Despite original studies highlighting *Toxoplasma* transmission via the consumption of undercooked foods (Desmonts *et al.*, 1965) this route of transmission could not account for the large number of infections in vegetarians (Jacobs *et al.*, 1963). Tachyzoites have been found in the milk of sheep, goats and cows (Jackson and Hutchison, 1989, Evans, 1992, Teneter *et al.*, 2000) however, transmission via consumption of these products has so far remained unproven with the exception of unpasteurised goats milk. Furthermore, consumption of contaminated drinking water can result in infection as observed by periodic outbreaks of Toxoplasmosis (Benenson *et al.*, 1982).

Isolation of viable parasites from the faeces of cats led to the discovery of faecal-oral transmission of *T. gondii* (Hutchison, 1965). Accidental contamination of food or drinks with infected faecal material as a result of poor hygiene, can result in parasite transmission via the faecal-oral route with a period lasting between 5 and 20 days following infection normally elapsing before symptoms are displayed (Jones *et al.*, 2003).

Finally, *T. gondii* can be transmitted vertically from mother to child during pregnancy. The first case of congenital toxoplasmosis was confirmed in a new born girl in New York, by Wolf *et al.*, (1939) when the infant showed symptoms of convulsive seizures and lesions in the eyes. Following the death of the child at 1 month, the presence of both intracellular and extracellular *T. gondii* tachyzoites were confirmed. Since then, research has shown that acute infection of a mother during pregnancy can result in the parasite being able to cross the placenta and establish congenital infection (Reviewed Jones *et al.*, 2003). The mechanism by which this vertical transmission occurs is not yet fully understood. However, it is generally believed tachyzoites replicate and disseminate into the foetal circulation (Remington *et al.*, 1990).

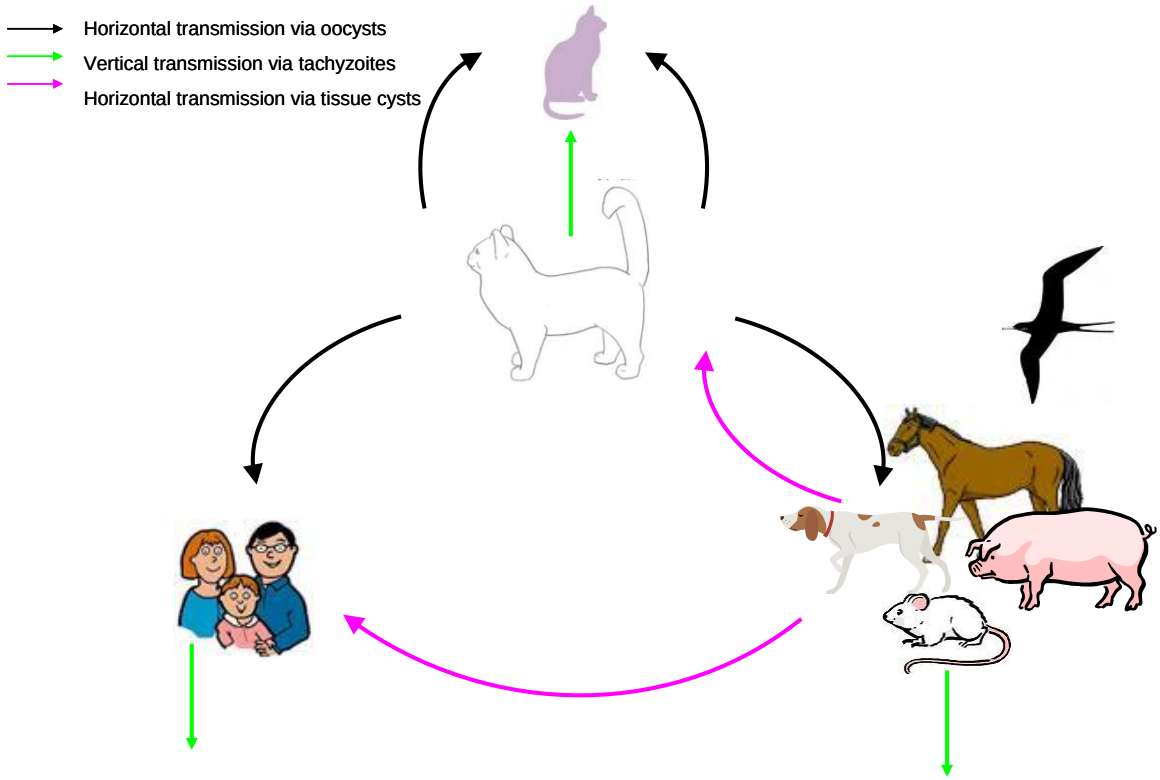


Figure 1.3: Transmission of *T. gondii*

Toxoplasma can be transmitted from cats to humans following accidental ingestion of infective oocysts in cat faeces. *T. gondii* can be transmitted from other intermediate hosts to humans via the consumption of undercooked meats in which tissue cysts may not have been successfully destroyed. Vertical transmission is possible from mother to foetus in both the intermediate host and definitive host.

1.4 Disease Manifestations

1.4.1 Immunocompetent Individuals

In immunocompetent individuals, toxoplasmosis is generally chronic and asymptomatic and as a result, goes largely undiagnosed. Occasionally non specific flu like symptoms persist in infected individuals such as muscle aches and pains, fever and malaise (Lu *et al.,* 2004). However, normally infection is self limiting and does not require treatment (Rorman *et al.,* 2006).

1.4.2 Immunocompromised Individuals

In immunocompromised patients such as HIV patients, infection with *T. gondii* is relatively common with 18-25% of AIDS patients in the US suffering from toxoplasmic infection at some point throughout their illness (Kasper and Gatel, 1998). Infection with *T. gondii* can be life threatening to HIV patients, often with the development of severe disease manifestations involving the central nervous system (CNS). Lesions are often formed in the brain resulting in the development of toxoplasmic encephalitis (TE) which is often fatal, if not treated, in these patients (Wijdicks *et al.,* 1991). Fatality is usually caused by reactivation of a latent infection whereby bradyzoites convert into rapidly dividing tachyzoites whose growth cannot be controlled (Bertoli *et al.,* 1995, Luft and Remington, 1992). Such events cause the pathological changes associated with TE (Kasper and Gatel, 1998).

Immunosuppressed transplant patients are also at risk of severe *T. gondii* infection from reactivation of a latent infection as well as the possible introduction of a new *T. gondii* strain from the transplanted organ. The suppressed immune response in these individuals usually struggles to overcome infection and consequently severe infection can result (Rorman *et al.,* 2006).

Congenital Toxoplasmosis

Congenital toxoplasmosis is caused by the vertical transmission of the parasite from a seronegative mother who becomes acutely infected with *T. gondii* during pregnancy. Infection before pregnancy is highly unlikely to result in congenital transmission. The occurrence of congenital toxoplasmosis varies greatly worldwide. For example, in France there are 4900 cases of primary *Toxoplasma* infection in pregnant women every year (Baril *et al.,* 1999) whilst in the UK there is infection in approximately 10

neonates per 10,000 women (Allain *et al.,* 1998). As a result, screening of pregnant women for infection is routine in France and Austria but not in the U.S.A and U.K. where prevalence is lower (Reviewed Kravetz and Federman, 2005).

The time at which the expectant mother becomes infected during pregnancy is critical to the outcome of infection (Dawson *et al,* 2005). Infection in the first trimester can result in abortion, mental retardation or hydrocephalus of the foetus whereas infection in the third trimester results in less severe or asymptomatic infection of the foetus and a low risk of abortion (Nimni *et al.,* 2004). It has been suggested that abortion, most frequently observed in the first trimester, is mediated by a Th1 response induced by the parasite that is incompatible with pregnancy. On the other hand, efficient transmission observed in the third trimester is due to a pregnancy induced Th2 response which is incompatible with controlling parasite multiplication. Therefore, the frequency of vertical transmission increases with progression of the gestation period (Dunn *et al.,* 1999). In any subsequent pregnancy of the same woman, the new foetus is generally not at risk of infection.

Ocular Toxoplasmosis (OT)

OT can be either congenitally or post natally acquired in both immunocompetent and immunosuppressed individuals (Vallochi *et al.,* 2002; Lu *et al.,* 2004). Some studies would suggest that postnatal and congenital infections are influenced by geographical location. For example, in Southern Brazil most cases of OT are postnatal whereas in the United States the majority of cases are congenital (Vallochi *et al.,* 2002). This difference is most likely due to the atypical strains that are predominant in South America (See chapter 1.1).

There are reported incidences of OT in apparently healthy people resulting in inflammation of the retina and uveal tract which can account for up to 20% of infected individuals (Vallochi *et al.,* 2002; Lyons *et al.,* 2001) and indeed active retinochoroiditis accounts for 0.4 cases/ 100,000 of the population in London, however, the number of cases of inactive retinochoroiditis is unknown (Gilbert *et al.,* 1995). Toxoplasmic retinochoroiditis usually presents at the posterior pole in a single eye with the presence of lesions which can result in retinal scarring (Commodaro *et*

al., 2009). Subsequently, *T. gondii* infection is the most common cause of infectious retinochoroiditis in immunocompetent individuals.

OT can also be a consequence of congenital infection. Cases of OT can result in a loss of both eye sight and hearing as well as other symptoms associated with congenital infection (Montoya and Remington, 2008). Initially congenital infection was believed to account for the majority of cases of OT in the UK (Perkins, 1973). However, it is now known that most cases of OT in the UK are due to postnatal infection (Holland, 1999, Gilbert and Stanford 2000), a pattern which is observed in other countries such as Brazil (Glasner *et al.*, 1992).

1.5 Treatment

Treatment for OT has remained consistent for many years and is somewhat limited to the same standard anti-parasitic drugs: pyrimethamine and sulphonamides (Rothova *et al.*, 1998). Classically, both pyrimethamine and sulphadiazine can be given in combination with corticosteroids to ease inflammation of the retina and other tissues of the eye (Rothova *et al.*, 1998, Montoya and Liesenfeld, 2004). However, a recent report by Commodaro *et al.*, (2009) indicates that combination therapies of corticosteroids with pyrimethamine, sulphadiazine and clindamycin does not reduce inflammatory activities. Therefore, patient response to antibiotic therapy in concert with corticosteroids varies greatly which may be a result of the hosts immune response (Vallochi *et al.*, 2002).

Antenatal treatment of congenital toxoplasmosis can be either with the administration of spiramycin or pyrimethamine sulphadiazine. However, their effectiveness is often debated in the literature. Ricci *et al.*, (2003), demonstrated that mothers with susceptible pregnancies who had not been treated with spiramycin or pyrimethamine, are four times more likely to be at risk of giving birth to a child with congenital toxoplasmosis than if the mother had been given drug treatment. As a result, all pregnant women who are diagnosed with primary infection are given drug treatment.

For OT, treatment with a combination of both pyrimethamine and azithromycin results in severe side effects and consequently a multidrug treatment is considered the only acceptable treatment. However, many of these drugs are toxic with unpleasant

side effects and consequently there is an ongoing search for newer and more effective anti- toxoplasmic drugs (Black and Boothroyd, 2000). Prime candidates include the azalide antibiotics. Azalide antibiotics are unable to prevent recurrent events of toxoplasmosis, analogous to standard therapies, but crucially it is a non-toxic antibiotic which could have implications in administration to those individuals unable to tolerate standard drugs (Rothova *et al.*, 1998).

1.6 The Immune Response

The immune response to *T. gondii* infection is complicated and circumstantial. Indeed, differences are observed in response to infection which are dependent on parasite strain, route of infection and animal model studied or even tissue dependent therein. However, there are several common themes in immunity to *T. gondii* and these are discussed below (Figure 1.4).

1.6.1 Protective Immunity

A protective adaptive immune response develops rapidly in an immunocompetent host following infection with *T. gondii*. Consequently, any re-infection of this individual should be resolved quickly by the immune system. Maintenance of protective immunity is believed to be associated with the periodic ruptures of cysts releasing bradyzoites which consequently stimulates the expansion of the effector immune response as well as maintenance of memory T cells (Subauste and Remington, 1993).

1.6.2 Cellular Mediated Immunity (CMI)

IL-12 and IFN-γ are essential mediators of resistance to *T. gondii* infection

Infection with *T. gondii* results in the release of pro-inflammatory cytokines such as IL-12 and IL-6. In particular, the release of IL-12 from macrophages, dendritic cells (DC) and neutrophils (Gazzinelli *et al.*, 1993b, Reis e Sousa, 1997, Bliss *et al.*, 1999) has been shown to activate Natural Killer cells (NK cells) to produce IFN-γ (Kelly *et al.*, 1989). IL-12 is composed of two subunits, p35 and p40, and is essential for optimal IFN-γ production (Gazzinelli *et al.*, 1994). The production of IL-12 is initiated following *Toxoplasma* engagement of CCR5 (Aliberti *et al.*, 2000), TLR-2 (Mun *et al.*, 2003) or TLR-11 (Yarovinsky *et al.*, 2005) which then triggers signalling pathways through MYD88. Consequently both $CCR5^{-/-}$ and $MyD88^{-/-}$ mice have

significantly reduced levels of IL-12 and increased susceptibility to infection (Aliberti *et al.,* 2000, Scanga *et al.,* 2002). Furthermore, neutralisation of IL-12 results in increased susceptibility to *T. gondii* infection (Gazzinelli *et al.,* 1994).

The importance of T cell activity in *T. gondii* infection was highlighted in the 70's and 80's when an increase in the number of transplant, cancer and AIDS patients resulted in an increase in overt toxoplasmosis in these individuals. In particular, it was observed that reactivation of a previous infection in AIDS patients could lead to TE (Horowitz *et al.,* 1983, Pitchenik *et al.,* 1983). These observations, in addition to experiments whereby the deletion of $CD4^+$ and $CD8^+$ from mice infected with *T. gondii* resulted in TE, provided evidence for the importance of these cells in immunity (Denkers and Gazzinelli, 1998). $CD4^+$ T cells are responsible for the production of pro and anti-inflammatory cytokines, and consequently they are essential for a successful immune response to overcome infection. The importance of $CD4^+$ T cells was further emphasised by one study which showed that the development of resistance to *T. gondii* was mediated by $CD4^+$ T cells (Araujo, 1991). Indeed, a direct protective role is observed via $CD4^+$ T cells although, some studies have reported an immunopathological role for these cells. For example, $CD4^+$ cells have been implicated in being involved in initiating an inflammatory response which exacerbates tissue inflammation in both the eye and the brain during *T. gondii* infection (Israelski *et al.,* 1989, Lu *et al.,* 2004).

Therefore, T cells were shown to produce IFN-γ, in a IL-12 dependent manner, to activate macrophages to produce reactive oxygen intermediates (ROI) (Nathan *et al.,* 1983) with IL-12 being required to drive $CD4^+$ and $CD8^+$ cell proliferation (Reis e Sousa *et al.,* 1997). However, cell transfer studies in nude mice highlighted the importance of $CD8^+$ T cells in controlling *T. gondii* replication (Parker *et al.,* 1991). Indeed, primary immunity against *T. gondii* can be further mediated by $CD8^+$ cells producing IFN-γ. Activation of $CD8^+$ cells by Th1 cells secreting IL-2, results in cytotoxic activity against tachyzoites and host cells infected with intracellular *T. gondii* (Fux *et al.,* 2003). However, $CD8^+$ T cell immunity can be induced but not maintained in mice lacking $CD4^+$ T cells (Casciotti *et al.,* 2002). Therefore, overall it is the general consensus that the actions of both $CD4^+$ and $CD8^+$ cells in concert are required for successful resolution of infection and that the continued production of

IFN-γ from both cell types is necessary for maintenance of resistance (Gazzinelli *et al.,* 1991).

T. gondii was first shown to induce the production of IFN-γ by Freshman *et al.,* in 1966 and since then the critical role of IFN-γ in response to *T. gondii* infection has been well characterised. Indeed early reports identified IFN-γ as an important mediator of protection during infection (Suzuki *et al.,* 1988, Suzuki *et al.,* 1989) and furthermore IFN-γ knock out mice could not survive the acute stages of infection (Scharton-Kersten *et al.,* 1996). Subsequently, antibody studies also showed IFN-γ to be required for survival in both acute and chronic stages of infection (Gazzinelli *et al.,* 1992a). Production of IFN- γ by NK cells is dependent on the production and secretion of IL-12 and TNF-α from activated macrophages and on signalling pathways such as STAT1 and STAT4 (Filisetti and Candolfi, 2004, Nagineni and Hooks, 2002). IFN-γ produced by NK cells in synergy with endogenous TNF-α activates macrophages and induces an increase in phagocytosis of tachyzoites (Sher *et al.,* 1993). Studies elsewhere have also demonstrated that neutralisation of IFN- γ and TNF-α, results in reactivation of disease from a previous infection confirming their importance in mediating resistance (Gazzinelli *et al.,* 1993a, Gazzinelli *et al.,* 1996) and in the production of toxic free radicals and nitric oxide (NO).

1.6.3 The role of innate immune cells in *T. gondii* infection

Macrophages

The importance of the macrophage in *T. gondii* infection was first highlighted in 1972, when Remington *et al.,* demonstrated that macrophages were required to control infection. In particular, the production of NO from macrophages following stimulation with IFN-γ can directly kill tachyzoites by inhibiting mitochondrial and nuclear enzymes (Brunet *et al.,* 2001). High concentrations of NO are produced by the conversion of L-arginine to citrulline by iNOS in the presence of IFN-γ, TNF-α or IL-1β. However, the role for NO during infection is not clear cut. During acute infection, NO production can be detrimental to the host with immune pathology being observed in mice (Khan *et al.,* 1997). However, as NO has been described as a mediator in the stage conversion of tachyzoites to bradyzoites, NO may also be regarded in chronic infection as a factor which keeps the parasite dormant (Bohne *et al.,* 1994). In addition, the effect of NO production in different strains of mice is

apparent whereby C57/BL6 mice have a requirement for NO and BALB/c mice do not (Schluter *et al.,* 1999). Furthermore, NO modulates IFN-γ production in *T. gondii* infected C57/BL6 mice, however, this protective effect is not observed in the resistant BALB/c mice (Kang *et al.,* 2004).

Activated macrophages also produce anti-microbial ROI upon stimulation with IFN-γ. A variety of ROI are produced including hydrogen peroxide and the superoxide radical. Indeed, the superoxide anion can react with NO to form peroxynitrite which can disrupt cell membranes (Brunet *et al.,* 2001). However, the role of ROI in controlling infection is debated as early studies in human macrophages showed *T. gondii* was susceptible to ROI (Murray *et al.,* 1985), whereas the opposite was observed in murine macrophages (Chang and Pechere, 1989).

In addition to the production of NO, ROI and cytokines, macrophages can also clear parasitic infection by autophagy. Tachyzoites usually reside in a PV which is resistant to fusion of lysosomes, although, invasion of an activated macrophage can result in autophagosomal parasite elimination (Yap *et al.,* 2007). In mice, this process can be mediated via IFN-γ induced GTPases which degrade the PV (Taylor *et al.,* 2000, Martens *et al.,* 2005), although there is currently no evidence of IFN-γ induced GTPases in humans.

Neutrophils

Neutrophils are present in the bloodstream where they circulate for a few hours upon release from the bone marrow, after which point they die. Successful resolution of infection relies on the rapid migration of neutrophils to the site of *T. gondii* infection where they function to phagocytose invading pathogens (Bliss *et al.,* 2000). They have granules containing microbiocidal molecules e.g. ROI and enzymes and they are also a source of pro-inflammatory cytokines such as IL-12, TNF-α and macrophage inflammatory protein 1α (MIP-1α) and MIP-1β (Denkers *et al.,* 2004, Reviewed Filisetti and Candolfi, 2004) with MIP-1α and MIP-1β serving as mediators which recruit T cells, monocytes, macrophages and immature DCs. Generally, granulocytes produce cytokines in low concentrations. However, due to the large number of neutrophils in the circulation and the speed at which they migrate to the site of infection, the importance of neutrophils in mediating the immune response in early *T.*

gondii infection is clearly evident. Denkers *et al.,* (2004), have shown that the deletion of neutrophils and other polymorphonucleocytes early in *T. gondii* infection, results in mice succumbing to infection. However, removal of neutrophils later in infection has no consequence on disease outcome (Bliss *et al.,* 2000). Furthermore, an influx of neutrophils to the site of infection has been shown to be dependent of CXCR2 and CCR1 (Del Rio *et al.,* 2001, Khan *et al.,* 2001), both of which are high affinity receptors for IL-8 in humans and MIP-2α in mice (Lee *et al.,* 1995). In addition, neutrophil development and recruitment is dependent on IL-17 as IL-17$^{-/-}$ mice have higher parasite burdens and reduced neutrophil recruitment. Indeed, neutrophil induction during acute infection has been shown to be dependent on IL-17 signalling whereby defective signalling results in mice succumbing to acute infection (Kelly *et al.,* 2005).

Dendritic Cells

The inflammatory response initiated following infection is further regulated by DC during *T. gondii* infection. DCs migrate to the site of infection following a chemokine signal secreted by neutrophils where they acquire foreign antigen. DCs then migrate to the spleen where they accumulate in T cell dense areas to present antigen to T cells. Such activity results in the promotion of a type-1 response driven by the production of IL-12 (Reis e Sousa *et al.,* 1997, Gubbels *et al.,* 2005). Subsequently, DCs are a major source of IL-12 although secretion is dependent upon two signals; microbial stimuli and CD40 – CD40L interaction (Seguin and Kasper 1999). This is a tightly controlled mechanism to the extent DCs have the ability to differentiate between living and dead tachyzoites. Subauste and Wessendarp (2000) demonstrated that only living tachyzoites induce up regulation of CD40 and IL-12 synthesis from DCs, which can subsequently initiate T cell IFN-γ release. Furthermore, DC produced IL-12 is required for protection from parasite reactivation in chronic infection (Yap *et al.,* 2000) and consequently DC IL-12 production has since been shown to be essential for resistance during *in vivo* infection. The production of IL-12 by DC is driven via TLR signalling which leads to activation of MyD88 whereby MyD88$^{-/-}$ mice have a significant reduction in IL-12 production upon challenge with *T. gondii* (Scanga *et al.,* 2002).

Recently, studies have reported exploitation of DCs by *T. gondii* in order to facilitate parasite dissemination. Currently, the precise mechanisms by which *T. gondii* successfully disseminates throughout the body are unknown. Indeed, in mice infected orally with *T. gondii,* tachyzoites can be found in the spleen and liver only a few hours after infection (Dubey, 1998a). Since then, evidence has suggested that DCs serve as a vehicle for parasite dissemination. *T. gondii* infected human monocyte derived DCs and murine bone marrow derived DCs have greater hypermotility compared with uninfected DCs which may potentiate dissemination (Lambert *et al.,* 2006). Subsequent studies have also suggested that in doing this, *T. gondii* may also suppress the DC IL-12 producing function (Bierly *et al.,* 2008).

1.6.3 Regulatory mechanisms in controlling responses to *T. gondii* infection

A balance is required between protective immunity provided by pro-inflammatory responses and immune pathology which can result from an excessive inflammatory environment. IL-10 is an important regulatory cytokine which is produced by DCs, macrophages and type 2 T cells and regulatory T cells (Moore *et al.,* 2001, Levings *et al.,* 2002). Despite historically being described as an inhibitor of type-1 responses, more recently an inhibitory role for IL-10 in type-2, T regulatory and Th17 responses has been described (Moore *et al.,* 2001, Reviewed Lieberman and Hunter, 2002, O'Garra and Vieira 2007). Furthermore, IL-10 also can inhibit the production of cytokines such as TNF-α and IL-12 from accessory cells which are required for optimal production of IFN-γ from T cells and NK cells, which consequently results in a decrease in IFN-γ (Fiorentino *et al.,*1991, reviewed Moore *et al.,*1993). However, early experiments indicated that IL-10 antagonised IFN-γ primed macrophages to kill intracellular tachyzoites (Gazzinelli *et al.,* 1992b). Reports have also suggested that IL-10 can also exert its regulatory function by down regulating MHC and co stimulatory molecules on macrophages whilst also influencing macrophage effector function by down regulating NO production (Gazzinelli *et al.,*1992b). Indeed, the regulatory role of IL-10 is underlined in experiments which have shown that IL-10 deficient mice die during acute infection due to excessive IL-12 and IFN-γ production (Gazzinelli *et al.,* 1996). However, the source of IL-10 is poorly understood. Some studies have reported the production of IL-10 from peritoneal macrophages in response to the virulent RH (Bliss *et al.,* 2000), whereas others have documented that

B-2 type B cells are a source of IL-10 (Mun *et al.,* 2003). However, the most important source of immunoregulatory IL-10 is believed to be $CD4^+$ T cells (Roers *et al.,* 2004). Unexpectantly, IFN-γ secreting $Tbet^+FoxP3^-$ Th1 cells are also major producers of IL-10. These cells are subsequently capable of exerting potent anti parasitic effector functions whilst suppressing IL-12 production from APC. This suggests that IL-10 production may not involve a distinct regulatory T cell subset (Jankovic *et al.,* 2007). However, in the chronic stages of infection when the production of IFN-γ is necessary to prevent reactivation of previous infection, very little is known of the exact role of IL-10. This is a somewhat controversial area as Deckert-Schluter *et al.,* (1997) reported that IL-10 caused increased parasite burdens in the brain whereas more recently Wilson *et al.,* (2005) demonstrated that a presence or absence of IL-10 did not affect parasite burdens in the CNS.

A regulatory role for IL-27 has also been described. IL-27 shares sequence and structure similarity to IL-12 and has the ability to enhance IFN-γ production, with initial studies indicating a role for this cytokine in Th1 responses (Trinchieri *et al.,* 2003). However, one study demonstrated that IFN-γ production by $CD4^+$ and $CD8^+$ cells in $IL\text{-}27R^{-/-}$ mice remained unchanged one week following infection, which led to the suggestion that IL-27 may influence Th1 polarisation in situations where IL-12 is limiting, but when in abundance, as in *T. gondii* infection, IL-27 is immunosuppressive (Villarino *et al.,* 2003). Other studies have demonstrated that IL-27 mediates negative regulation of Th1 polarised responses by inhibiting the production of IL-2, a potent T cell growth factor. (Villarino *et al.,* 2006). This data led to the conclusion that in cases of acute *T. gondii* infection, the ability of IL-27 to direct Th1 responses becomes secondary to the negative regulatory role of IL-27 in suppressing T cell functions (Hunter *et al.,* 2004). In addition to a role for IL-27 in type-1 responses, other studies have described anti-inflammatory properties of IL-27 type-2 and Th 17 responses (Rosas *et al.,* 2006, Artis *et al.,* 2004, Batten *et al.,* 2006). Indeed, a recent study by Stumhofer *et al.,* (2006) highlighted the ability of IL-27 as a suppressor of Th17 differentiation of naïve $CD4^+$ T cells, in response to *Toxoplasma* antigens. Expansion of Th17 cells occurs in the presence of TGF-β, IL-6 and IL-23 which promote pro-inflammatory, anti-microbial activity by secretion of cytokines such as IL-17. As discussed previously, IL-17 is an important mediator for neutrophil development and therefore an important role for this cytokine has been outlined

during *T. gondii* infection. Therefore, taking into account the suppressive effects of IL-27 on Th17 cells, a role for IL-27 in regulating T cell responses, similar to those of IL-10 and TGF-β, has been suggested with particular attention being focused on Th17 responses (Reviewed Gaddi and Yap, 2007).

1.6.4 Humoral Immune Response

Antibodies play a minor role in the immune response against *T. gondii* infection in comparison to elements of CMI. The humoral immune response to *T. gondii* is characteristic of that expected by invasion of any pathogen in that IgM is produced in the first 7 days following infection. The structure of IgM makes it a good complement activator and can undertake agglutination of parasites as well as being cytotoxic. Indeed, a study involving $IgM^{-/-}$ mice demonstrated an important role for IgM in acute *T. gondii* infection. In particular, parasite specific IgM was shown to limit *T. gondii* dissemination (Couper *et al.,* 2005). However, susceptible C57/BL6 mice have reduced levels of IgM 7 days post infection than resistant A/J mice (M^{c}Leod *et al.,* 1989). Following IgM secretion, IgG is produced; primarily IgG1,G2 and G3, which are capable of antibody dependent cytotoxicity (ADCC) and can also confer protection to an unborn foetus as these immunoglobulins of the IgG lineage are transplacental (Nimni *et al.*, 2004).

Furthermore, the ability of the parasite to enter host cells in less than 30 seconds (Sibley and Andrews, 2000) makes them less vulnerable to the actions of antibodies. However, during the blood stages of the parasite life cycle, antibodies provide short term protection against infection (Reviewed Filisetti and Candolfi, 2004). In addition, several studies have indicated an important role for the production of parasite specific antibodies by B cells in persistent infection of tachyzoites in mice (Kang *et al.*, 2000, Sayles *et al.,* 2000).

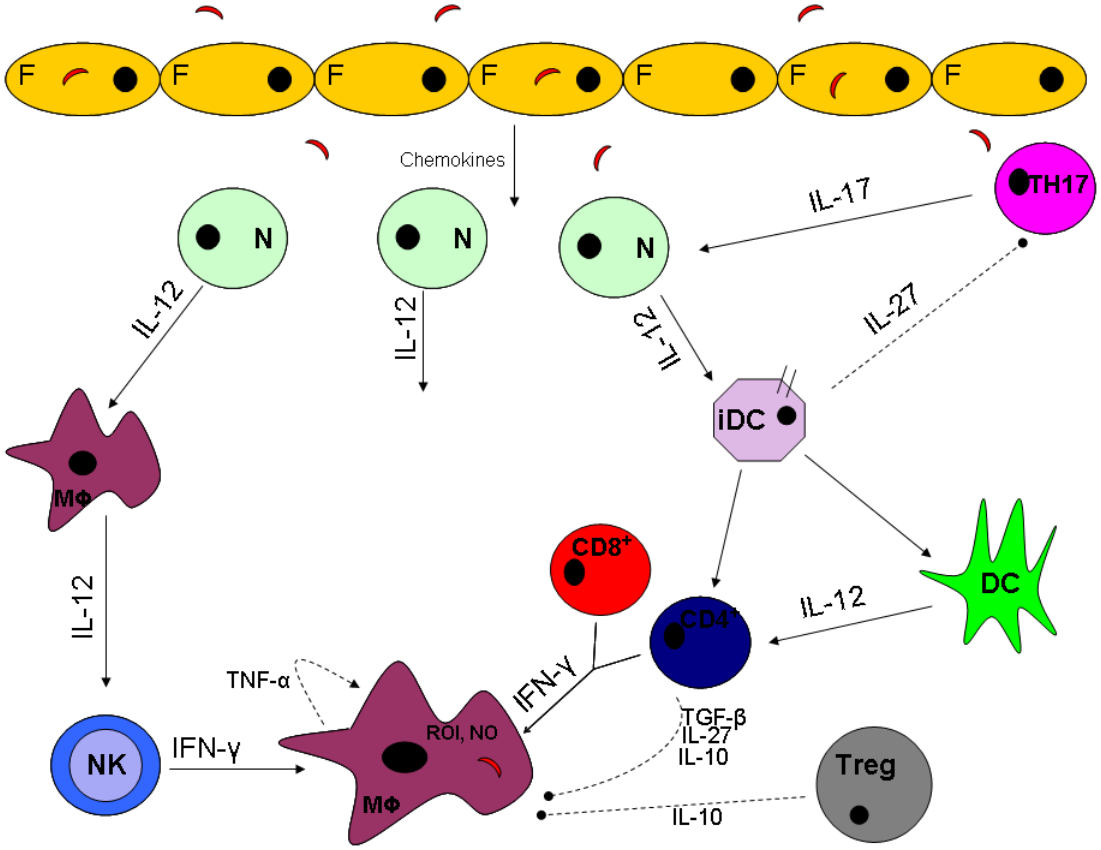


Figure 1.4: The immune response to *T. gondii* infection

Following infection with *T.gondii*, elements of both the innate and adaptive immune responses act to control infection. In brief, the production of IL-12 by cells such as neutrophils, dendritic cells, macrophages and $CD4^+$ (Th1) cells is required to activate NK cells and $CD4^+$ and $CD8^+$ T cells to produce IFN-γ. IFN-γ can activate macrophages, in association with TNF-α, to produce reactive oxygen intermediates (ROI) and nitric oxide (NO), which are associated with parasite killing. This pro-inflammatory response is regulated by cytokines such as TGF-β, IL-10 and IL-27 by $CD4^+$ (Th2) cells and T regulatory cells. Regulation of the pro-inflammatory response is required to prevent immune pathology which is often associated with an over exuberant Th1 response.

1.7 Establishment of *T. gondii* infection

T. gondii is an intracellular parasite which similar to other Apicomplexans, migrates to and from tissues and invades cells by a process known as gliding motility (Keeley and Soldati, 2004, Sibley, 2004). This process involves the interaction of actin filaments with myosin and gliding associated proteins which forms a connection with the parasite's inner membrane complex (Gaskins *et al.,* 2004). The actin then forms a connection with aldolase which promotes the connection of this motor structure to transmembrane proteins across the plasma membrane (Jewett and Sibley, 2003). Following this engagement, transmembrane adhesins are then released in a unidirectional fashion from the apical end of the parasite with forward movement being supported by actin microfilaments (Reviewed Carruthers and Boothroyd, 2007). Following detection of a new target cell, contact must be made between the parasite and cell for successful invasion. Studies to date have indicated that out of the many different proteins which coat the *T. gondii* surface, 6 may be implicated in the attachment and invasion process. These are surface antigen SAG proteins 1-3 and SAG related sequences 1-3 (Lekutis *et al.,* 2001). The importance of these proteins was determined using gene knock out and antibody inhibition studies. In one example, SAG3-deficient parasites had a 50% reduction in attachment (Dzierszinski *et al.,* 2000). Consequently, the first interactions between *Toxoplasma* and the host cell are a result of a series of low affinity interactions between SAG proteins and the host cell membrane. However, attachment of the apical end of the parasite to the host cell is mediated via microneme proteins which are secreted from the apical end in a calcium-dependent manner (Carruthers *et al.,* 1999). Deletion of selected microneme genes results in a reduction in efficiency of the invasion process (Huynh *et al.,* 2004, Cerede *et al.,* 2005) whilst simultaneous deletion of two MIC proteins results in a decrease in lethality of infection (Cerede *et al.,* 2005). The final stage in attachment involves another microneme protein called apical membrane antigen (AMA1) which forms a connection with rhoptry neck proteins to form a moving junction (MJ) (Alexander *et al.,* 2005, Lebrun *et al.,* 2005). The MJ is a small ring like structure which forms a close binding surface with the host cell. As the parasite invades the host cell, the MJ moves from the front to the back of the parasite with some reports indicating a sieve like function for the MJ which acts to exclude some host and parasite proteins (Charron *et al.,* 2004). Upon entry to the cell, the parasite then forms a PV (Suss-Toby *et al.,* 1996). The PV membrane consists primarily of

invaginated host membrane which over a period of time becomes pinched off. Subsequently, the composition of the PV becomes a mixture of extracellular medium and vacuolar space. Ultimately the invasion process has no effect on host cell integrity. The PV is resistant from fusion with host cell endosomes and lysosomes. However, Zhao *et al.,* (2009) have reported that, in mice, the PV can be ruptured by host interferon inducible immunity related GTPases, which results in parasite death. Despite this, during the normal course of infection, tachyzoites reside in the PV where they replicate before egress from the cell. Parasite egress can be via the action of *Toxoplasma* perforin like protein 1 which aids pore formation (Kafsack *et al.*, 2009) or alternatively by mechanical rupture of the host cell as studies have shown that blocking parasite motility does not prevent parasite exit from the cell (Lavine and Arrizabalaga, 2008).

1.8 *T. gondii* cell biology and protein secretion

T. gondii is often considered the model organism for studies on the Apicomplexa due to the ease at which it can be cultured and genetically modified (Roos *et al.,* 1994). In addition, the polarised organisation of *T. gondii* secretory organelles make it a desirable organism for studying secretory processes within the Apicomplexa (Hager *et al.,* 1999). Broadly speaking, the endoplasmic reticulum (ER) is concentrated posterior to the nucleus in *T. gondii*, which is in contrast to mammalian cells, where the ER is distributed throughout the cell. The golgi apparatus is found anterior to the nucleus and consequently, *T. gondii* is one of the few eukaryotes which use the nuclear envelope as an intermediate between the ER and golgi (Rossanese *et al.,* 1999). Analysis of the *T. gondii* genome and EST databases have shown COPI and COPII coat components (Ajioka *et al.,* 1998) and subsequently a role for these proteins in the transport process of secretory proteins from the ER to golgi has been suggested. Typically, from the golgi, proteins are transported in clathrin coated vesicles from the trans golgi stack to one of three specialised secretory organelles associated with Apicomplexa. Alternatively, those proteins destined for the parasite surface are delivered by an unusual route apparently involving GPI anchors as targeting motifs (Karsten *et al.,* 1998).

As found in all Apicomplexan parasites, *T. gondii* has three distinct sercetory organelles: micronemes, rhoptries and dense granules. These specialised organelles

function in a sequential fashion with micronemes being triggered first to initiate host cell penetration followed by rhoptry secretion which is essential for parasite survival in the host cell. Although dense granule proteins are constitutively expressed, they are required for the maintenance and intracellular replication of the parasite (Carrurthers and Sibley 1997). Collectively, these organelles work together to maintain parasitic function and survival.

A schematic of the *T. gondii* tachyzoite cell biology is shown in Figure 1.5.

1.8.1 Dense Granules

Dense granules are distributed throughout the cell and are largely indistinguishable from secretory granules present in other cell types and their secretion is both constitutive and calcium independent (Karsten *et al.*, 1998). The dense granules are considered to be the default pathway for all soluble proteins (Karsten *et al.,* 1998). Therefore dense granule proteins are signal independent and even proteins destined for other secretory organelles are automatically routed to the dense granules following the deletion of targeting signal sequences (Striepen *et al.,* 1998, Reiss *et al.,* 2001). To date, 13 GRA proteins have been identified including 9 specific GRA proteins, 2 nucleoside triphosphate hydrogenases and 2 protease inhibitors. GRA proteins are relatively small, ranging from 21-41KDa and posses little homology with any other protein of known function (Braun *et al.,* 2008). Typically, dense granules fuse with the plasma membrane and constitutively release their contents into the PV (Karsten *et al.*, 1998). Upon secretion from the apical tip, dense granule proteins can either become associated with the PV membrane or alternatively they can become integrated into the tubular network. For example, GRA 2 in association with GRA 6 has been shown to be involved in the formation of a membranous network (Mercier *et al.*, 2002).

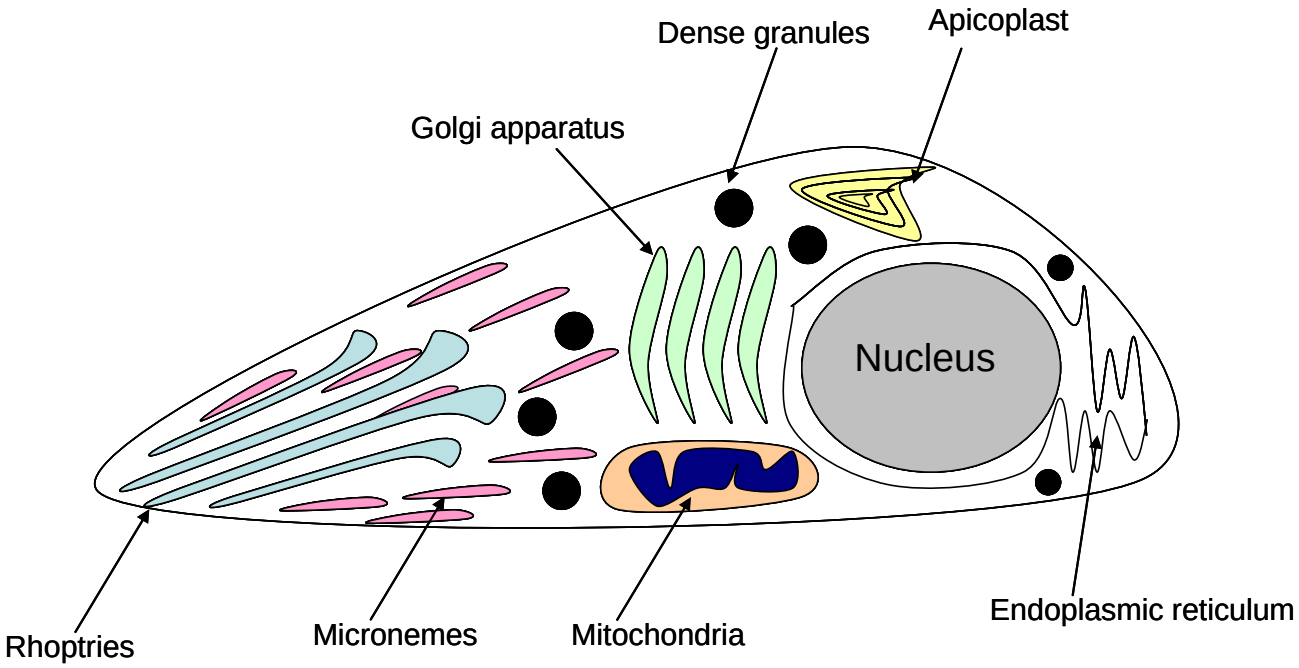


Figure 1.5: A diagrammatic representation of a *T. gondii* tachyzoite

The cell biology of the tachyzoite is shown above. The endoplasmic reticulum is primarily concentrated to a position which is posterior to the nucleus, whereas, the golgi body is found anterior to the nucleus. The organisation of secretory organelles is highly polarised in *Toxoplasma*, with rhoptries and micronemes being located at the anterior end of the parasite whereas dense granules are distributed throughout. Diagram based on Ajioka *et al.,*2001.

1.8.2 Rhoptries

Rhoptries are distinct, acidified secretory organelles, of which there are approximately 8-12 per parasite (Ngo and Joiner, 2004). They are located in an anterior position in the cell (Joiner and Roos, 2002) and are involved in the formation of the PV. To date, over thirty major rhoptry proteins have been identified in *T. gondii* (Dlugonska, 2008). Targeting of rhoptry proteins is via multiple independent signals (Bradley and Boothroyd, 2001). For example, members of the ROP2 family of proteins exhibit a YXXΦ motif within the cytoplasmic tail which when deleted or mutated results in the exit of ROP2 from the trans-golgi network. Consequently, delivery of mutated proteins to the mature rhoptries in *T. gondii* is significantly reduced (Hoppe *et al.*, 2000). It has been suggested that during routine targeting of rhoptry proteins, proteins are transported as part of the endosomal pathway from the golgi apparatus to a precursor compartment, prior to the final targeting of proteins to the rhoptries (Robibaro *et al.*, 2002).

Secretion of proteins from the rhoptries results in the formation of the parasitophorous vacuole membrane (PVM), aiding parasite survival within the host cell by protecting the parasite from the host endocytic pathways. In particular, ROP2 is secreted and inserted into the PVM (Beckers *et al.*, 1994). The high cholesterol content found in rhoptries had initially suggested a role for these proteins in lipid bilayer stability. However, this was quickly dismissed due to a high cholesterol/phospholipids ratio (Foussard *et al.*,1991). As a result, cholesterol is thought to be a non essential component of the PVM (Ngo and Joiner, 2004).

In addition to ROP proteins, the rhoptries also secrete proteins from the neck region; these are rhoptry neck proteins (RON). RON4 was the first rhoptry neck protein to be identified and a role for this protein in MJ formation was first described. Specifically, RON4 forms an interaction with AMA1 whereby they both localize at the MJ during invasion (Alexander *et al.*, 2005, Leburn *et al.*, 2005). Similarly, a role for RON2 in the formation of the MJ as also been described (Boothroyd and Dubremetz, 2008). Indeed, RON proteins are found in other Apicomplexa including *Plasmodium*, thus suggesting an important biological role for these proteins in host cell invasion (Cao *et al.*, 2008).

1.8.3 Micronemes

Micronemes, like rhoptries, are generally located at the anterior end of the cell and are involved in host cell adhesion. Like rhoptry proteins, microneme (MIC) proteins can possess multiple targeting domains (Striepen *et al.,* 2001) and are involved in the recognition and adhesion to a host cell. Secretion from the micronemes is initiated by physiological triggers and indeed Ca^{2+} mediated microneme secretion results in motility and host cell penetration. MIC proteins also have escort roles whereby they facilitate the targeting of other MIC proteins e.g. MIC6 escorts microneme proteins MIC1 and MIC4 by forming a trimeric complex, whereby deletion of MIC6 prevents targeting of MIC1 and MIC4 to the micronemes (Reiss *et al.,* 2001). Many *T. gondii* microneme proteins do not possess a transmembrane domain and YXXΦ motif, however targeting to micronemes is thought to be via a tyrosine-dependent process (Hoppe *et al.,* 2000). For example, MIC2 is predicted to contain a transmembrane domain with a cytoplasmic tail encoding tyrosine motifs, that are believed to play a role in the sorting process (Di Cristina *et al.,* 2000).

1.8.4 Apicoplast

The Apicoplast, a relict plastid, is one of the most unusual organelles found within *T. gondii* and indeed other Apicomplexa. It was acquired via secondary endosymbiosis and retention of a eukaryotic alga (Kohler *et al.,* 1997) and subsequently this plastid has become essential for *T. gondii* survival (Fichera and Roos, 1997; He *et al.,* 2001) with a suggested role in lipid metabolism (Waller *et al.,* 1998). As demonstrated by Waller *et al.,* (1998), apicoplast proteins are generally synthesised on cytoplasmic ribosomes and then post-translationally imported across the four membranes encompassing the apicoplast, with further suggestion of an NH_2 signal sequence. Therefore, proteins targeted to the apicoplast have an N terminal bipartite signal which is composed of a classical secretory signal which facilitates entry to the ER and secondly, a transit peptide to cross the inner membranes of the plastid (Waller *et al.,* 1998, Waller *et al.,* 2000).

1.9 *T. gondii* immune modulatory mechanisms

Many studies have detailed the ability of *T. gondii* to modulate host immune responses in order to provide a favourable environment for parasite survival. One such example was highlighted by Brenier-Pinchart *et al.,* (2000) who demonstrated the effects of *T. gondii* on host monocyte chemotactic protein (MCP). MCP is a CC chemokine which attracts and activates monocytes/macrophages whilst also activating arms of the CMI including $CD4^+$ and $CD8^+$ T cells. Fibroblasts were infected with tachyzoites and subsequently, supernatants were analysed for MCP-1. Uninfected control fibroblasts showed basal levels of MCP-1, however, an increase in MCP-1 was observed in tachyzoite infected fibroblasts. The authors suggested that the upregulation of MCP-1 may contribute towards the recruitment of monocytes and lymphocytes which ultimately control *T. gondii* infection. Nevertheless, infection of fibroblasts with live tachyzoites did not alter expression of IL-8 at the mRNA level 24 hours post infection. These results were similar to those of Friedland *et al.,* (1993) who demonstrated phagocytosis of *T. gondii* by THP-1 cells did not result in an increase of IL-8 production from these cells up to 24 hours post infection. This suggested the existence of parasite regulated mechanism which functioned to minimise the possibility of host cell intracellular killing mechanisms.

A subsequent study by Denney *et al.,* (1999) recorded an increase of both IL-8 and MCP-1 between 24 and 48 hours post infection of Hela cells and fibroblasts. Of note, IL-8 production was only observed when live tachyzoites were used in experimentation. Further studies have confirmed this finding whereby *in vitro* stimulation of THP cells with *T. gondii* lysate for 24 hours, resulted in an increase of IL-8 mRNA levels from basal levels (Jung-Lee *et al.,* 2008). Furthermore, co-treatment of cells with lysate and LPS resulted in a reduction of IL-8 from cells which had been stimulated with LPS alone but higher than IL-8 levels from cells treated with lysate alone. The authors suggest that these findings indicate a regulatory role being undertaken by the parasite to prevent an uncontrolled pro-inflammatory response which could ultimately lead to parasite elimination (Jung-Lee *et al.,* 2008)

Other studies have investigated *T. gondii* mediated alteration of host cytokine profiles. In one example, TNF-α expression from neutrophils following infection was analysed. Neutrophils have preformed stores of TNF-α which are released from the

cell surface. However, a cell bound form of TNF-α is also found on neutrophils, which is produced following TLR-4 stimulation, suggesting a potential cell-cell contact mediated mechanism of TNF-α action (Denney *et al.*, 1999). Subsequently, neutrophil stimulation with *Toxoplasma* lysate results in a reduction in TNF-α production in comparison to expression levels from those uninfected cells following TLR-4 activation. In addition, *T. gondii* tachyzoites have been shown in separate studies to alter LPS induced cytokine expression of TNF-α and IL-12 (Lee *et al.*, 2006) with suppression of such mediators aiding parasite evasion of NO and p47GTPase killing (Butcher *et al.*, 2005)

T. gondii mediated modulation of host immune responses have been described in the CNS where the maintenance of a chronic infection is reliant on IFN-γ which activates microglia to control parasite growth. However, a reduction in NO production is observed from IFN-γ driven microglia agent in the presence of infected astroctyes (Rozenfeld *et al.*, 2003). With NO being a potent anti-microbial, the benefits in restricting production of this mediator are clear.

1.9.1 Specific *T. gondii* proteins with immune modulatory properties

More recent studies have identified specific *T. gondii* proteins for which immune modulatory effects have been observed. Yarovinsky *et al.*, (2005) showed *T. gondii* profilin (TgPRF) was capable of inducing IL-12 production from DCs via activation of TLR-11. Although TLR-11 is found in the human genome, a premature stop codon in the coding sequence prevents protein expression. Despite this, TLR-11 is expressed in mice and indeed, TgPRF is the only described ligand for TLR-11 (Reviewed Lauw *et al.*, 2005). Subsequently, TgPRF has been aptly named as a 'danger' molecule i.e. one which alerts host immunity to the presence of a foreign pathogen. Infection of mice with parasites that do not express TgPFR results in a decrease in IL-12 production from splenic DC's (Plattner *et al.*, 2008). Indeed such parasites are still capable of attaching to host cells, however, the invasion process is significantly impaired. However, in order to validate the authenticity of these results being a direct result of gene deletion or indeed the ability of such knock out parasites to establish a sustained infection due to its inability to infect cells, the profilin gene of *P. falciparum* was introduced to the knock out. This transfection conferred infectivity of the knock out parasite whilst IL-12 levels remained low. However, the question

remains as to why such a ‘danger molecule’ has not been selected against through evolutionary selection from the *T. gondii* genome? Denkers and Striepen (2008) propose that optimal survival of *T. gondii* may require IL-12 production following TgPRF recognition as in the complete absence of IL-12 an environment favourable to uncontrolled parasite growth would occur and subsequently this could lead to both host and parasite demise.

Most recently, an immunomodulatory role was described for *T. gondii* cyclophilin 18 (TgCyp18). Previous studies have shown that TgCyp18 binding to CCR5 on DCs and macrophages upregulates IL-12 dependent production of IFN-γ (Aliberti *et al.,* 2003, Cai *et al.,* 2000). This ability of TgCyp18 to drive IL-12 production, led to studies investigating the effect of TgCyp18 on expression of other cytokines (Ibrahim *et al.,* 2009). Subsequently, recombinant TgCyp18 was shown to induce TNF-α and p40 IL-12 whilst further augmenting the levels of IL-6 and IFN-γ in a CCR5 independent manner. Furthermore, TgCyp18 increased NO production from stimulated macrophages, although, this activity was not reproducible following pre-treatment of cells with TgCyp18. Finally, TgCyp18 was shown to inhibit parasite growth in infected macrophages. These findings along with the IFN-γ and NO inducing properties of TgCyp18, prompted suggestion that TgCyp18 may be involved in tachyzoite to bradyzoite conversion. Indeed further experiments showed that TgCyp18 did induce the conversion process. Therefore, with this in mind, a role for TgCyp18 in modulating host immune responses has been suggested.

T. gondii derived heat shock protein 70 (TgHSP70) was first identified in 2000, when it was shown to be expressed at lower levels in immunocompromised mice infected with virulent RH (Miller *et al.,* 2000). Subsequently, the immunomodulatory properties of TgHSP70 were first characterised when Dobbin *et al.,* (2002) demonstrated that proliferation of virulent parasites with reduced HSP70 expression was significantly reduced *in vivo* whilst tachyzoite to bradyzoite conversion was increased. A later study also demonstrated the ability of TgHSP70 to drive differentiation of human monocyte derived DCs and IL-12 production (Kang *et al.,* 2004) whilst further studies have shown this activity to be mediated via TLR-4 in a MyD88 independent signalling pathway cascade (Aosai *et al.,* 2006). Furthermore, C57/BL6 mice succumbed to infection on day 9, following injection with TgHSP70

on day 3 post *T. gondii* infection (Ahmed *et al.*, 2004). Therefore, throughout the literature an important role for TgHSP70 has been outlined with some studies proposing that TgHSP70 functions to protect *T. gondii* from the anti-parasitic effects of the host immune response.

1.10 Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (MIF) was first identified in 1966 and consequently was one of the first cytokines to have been described. MIF was discovered in two independent studies, which described this molecule as a soluble mediator secreted from stimulated lymphocytes that inhibited migration of macrophages (Bloom and Bennett, 1966, David 1966). This was an unusual feature at the time and enthused many immunologists. Human MIF was not isolated and cloned until 1989, 23 years after its initial description (Weiser *et al.,* 1989). Soon after recombinant protein and anti-MIF antibodies were available for use and indeed localisation studies were some of the first to benefit from production of such immunological tools (Bernhagen *et al.,* 1993, Nishino *et al.,* 1995). However, the mechanisms by which MIF mediated its functional activity remains thus far somewhat obscure. In 1996, the structure of human MIF was solved (Sugimoto *et al.,* 1996, Sun *et al.,* 1996) illustrating that human MIF exists as a trimer with a structural architecture almost identical to a bacterial isomerase 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) (Figure 1.6). While initial experiments using gel filtration analytical ultracentrifugation techniques had suggested MIF may exist as a monomer or dimer (Galat *et al.,* 1994, Blocki *et al.,* 1992, Nishihira *et al.,* 1995), later studies confirmed the trimeric form to be the natural conformation of MIF (Lolis *et al.,* 1996, Mischke *et al.,* 1997, Bendrat *et al.,* 1997). Each MIF monomer comprising the trimer was estimated to have a MW 12.5KDa and to consist of two anti-parallel alpha helices packed against a four strand beta sheet. In addition, each monomer was shown to have two beta strands that interact with beta sheets of adjoining subunits (Sugimoto *et al.,* 1996, Sun *et al.,* 1996). The trimeric architecture of MIF also highlighted the formation of a pore, which has a positive charge of as yet unknown function (Sun *et al.,* 1996).

1.11 Human MIF gene and protein

The human MIF gene is 351bp long and consists of three introns and two exons (Paralkar and Wistow, 1994). The gene encodes a small protein which consists of 112 amino acids. Furthermore, it is known that the starting methionine is cleaved post translation to expose the N-terminal proline (Bernhagen *et al.,* 1993).

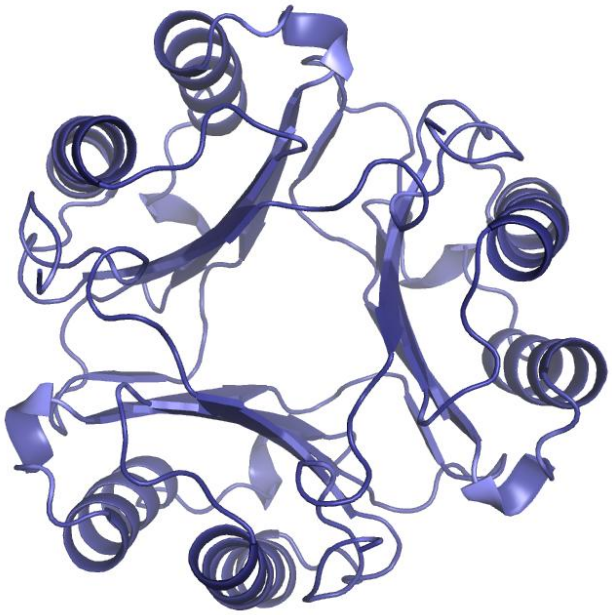


Figure 1.6 MIF 3D crystal structure

Mammalian MIF exists as a trimer with the formation of a central hydrophilic pore possessing a positive charge. The function of this pore is unknown (Lubetsky *et al.,* 2002).

An NCBI protein search of MIF retrieves results for over 20 mammalian species, 92 bacterial species and 16 different Apicomplexans. Published literature to date would suggest that all MIFs lack an N-terminal signal sequence for secretion via classical pathways (Flieger *et al.*, 2003) and recently a role for the golgi protein p115 has been suggested in the secretion of human MIF (Merk *et al.*, 2009).

1.12 Cell and tissue sources of mammalian MIF

While MIF is secreted by a huge variety of cell types, the main source of MIF is the macrophage where it is found preformed and secreted upon stimulation with LPS (Calandra *et al.*, 1994). *In vitro* the RAW264.7 macrophage line stimulated with IFN-γ and TNF-α, but not IL-6 or IL-1β secrete preformed MIF (Calandra *et al.*, 1994). However, studies demonstrate that MIF is also secreted from other immune cells such as T cells, B cells and eosinophils (Rocklin *et al.*, 1975, Calandra *et al.*, 1994, Rossi *et al.*, 1998). MIF mRNA is also found within the brain, where differences in mRNA levels are observed dependent on cell type, with neurons for example having increased MIF mRNA expression levels compared with glial cells (Bacher *et al.*, 2003). MIF is also secreted in pituitary corticotroph and thyrotroph cells, suggesting that MIF may have an endocrine role (Nishino *et al.*, 1995). Well-established roles for MIF have also been described from non-immune cells and tissues. For example, MIF is secreted, along with other pro-inflammatory cytokines from human adipose tissue during primary culture in the absence of any stimulants (Fain *et al.*, 2006). In addition, it is well documented that MIF is present in the reproductive system and secretion has been reported from uterine, endometrial and leydig cells (Schaefer *et al.*, 2005, Yang *et al.*, 2000, Meinhardt *et al.*, 1996).

1.13 The MIF Receptor

Until recently, the MIF receptor was unknown. Some studies initially suggested that MIF activation occurred via unconventional routes, such as an intrinsic catalytic activity (Hermanowski-vosatka *et al.*, 1999; Swope *et al.*, 1999; Kleemann et *al.*, 1998). However, in 2003, Leng *et al.*, described CD74 as a cell surface binding protein for MIF. CD74, like MIF, is a homotrimer (Jasanoff *et al.*, 1998) and is also known as the MHC class II invariant chain, which functions to transport class II proteins from the ER to the golgi complex (Cresswell, 1994). Approximately 2-5% of

cellular CD74 is expressed on the cell surface in many different cell types (Wraight *et al.,* 1990; Henne *et al*., 1995).

MIF is known to induce ERK-MAPK activation in macrophages, an activity which is reduced in $CD74^{-/-}$ macrophages (Kamir *et al.,* 2008). Furthermore, MIF increases ERK-MAPK phosphorylation in the human Raji B cell line, which expresses abundant cell surface CD74. An increase in ERK MAPK activation is associated with a significant increase in proliferation of Raji B cells; an effect which is diminished in the presence of anti-CD74 mAbs. This proliferative effect is not only observed in immune cells but also in cells such as fibroblasts (Lacey *et al*., 2003). However, the molecular dynamics of the MIF-CD74 interaction is unknown and indeed the mechanism by which signal transduction is initiated is currently unclear. As the short intracellular domain of CD74 lacks homology with tyrosine or serine/threonine kinases or interaction domains for non-receptor kinases, this prompted speculation that a second accessory protein was involved in the MIF receptor complex, which would facilitate signal transduction (Leng *et al.,* 2003, Shi *et al.,* 2006).

In pursuit of an accessory protein for CD74 in the context of a functional MIF receptor, Shi *et al.*, (2006) explored the possible role of CD44. A mutant cell line deficient in CD44 was incapable of MIF-mediated ERK phosphorylation, yet binding through CD74 was still facilitated. These results indicate that, through a process dependent on CD44, MIF induces serine phosphorylation of CD74 intracytoplasmic domain. This suggests that MIF binding to CD74 initiates ligand induced activation events which are essential for subsequent CD44 signal transduction.

Previous studies have detailed activation of ERK MAPK pathways via a Ras-Raf-MEK dependent pathway (Ishida *et al.,* 1998) following an association of c-Src (Src) kinases with the intracytoplasmic domain of CD44 (Taher *et al.,* 1996). Therefore, addition of Src inhibitors to cell lines which express both CD74 and CD44, resulted in inhibition of Src and ERK phosphorylation. Subsequently, these results suggest a mechanism by which MIF mediates signal transduction following interaction with CD74 (Shi *et al.,* 2006). A diagrammatic representation of the predicted mechanism by which signal transduction is initiated following MIF binding to its receptor is shown in Figure 1.7.

It is unlikely that all MIF biological activities are mediated exclusively via binding to CD74/CD44, as previous studies have demonstrated the ability of MIF to bind Jab-1 intracellularly, independent of a surface receptor. MIF binding to Jab1 results in decreased AP-1 gene activity thus enabling MIF to regulate gene expression (Kleemann et al., 2000). Furthermore, some target cells of MIF do not express CD74, such as neutrophils (Calandra and Roger, 2003) and consequently MIF mediated activity in such cells must be mediated via a different receptor or perhaps even a non-receptor-dependent mechanism. Subsequently, chemokine receptors CXCR2 and CXCR4 have been identified as binding partners for MIF (Bernhagen *et al.,* 2007). Specifically, MIF binding CXCR2 inhibited the random migration of monocytes whilst MIF binding CXCR4 resulted in the arrest of primary human effector T cells. Subsequently, MIF binding to CXCR2 and CXCR4 induced directed migration of monocytes and T cells respectively. In addition, this study demonstrated a physical interaction between CXCR2 and CD74, which formed a functional MIF receptor which induced leukocyte recruitment (Bernhagen *et al.,* 2007). In particular, a role for MIF in atherogenic recruitment in apolipoprotein E-deficient mice (*Apoe*$^{-/-}$) has been suggested to be mediated via CXCR2 and CD74. However, later studies showed that CXCR4 also formed a functional receptor complex with CD74 which is responsive to MIF (Schwartz *et al.,* 2009). Consequently, MIF binding to CXCR4/CD74 receptor complex resulted in CD74-dependent AKT activation in T cells which was inhibited by the addition of CXCR4 inhibitors. However, it is currently unclear whether a role for CD44 is evident in either CXCR2 or CXCR4 receptor complexes with CD74 (Schwartz *et al.,* 2009).

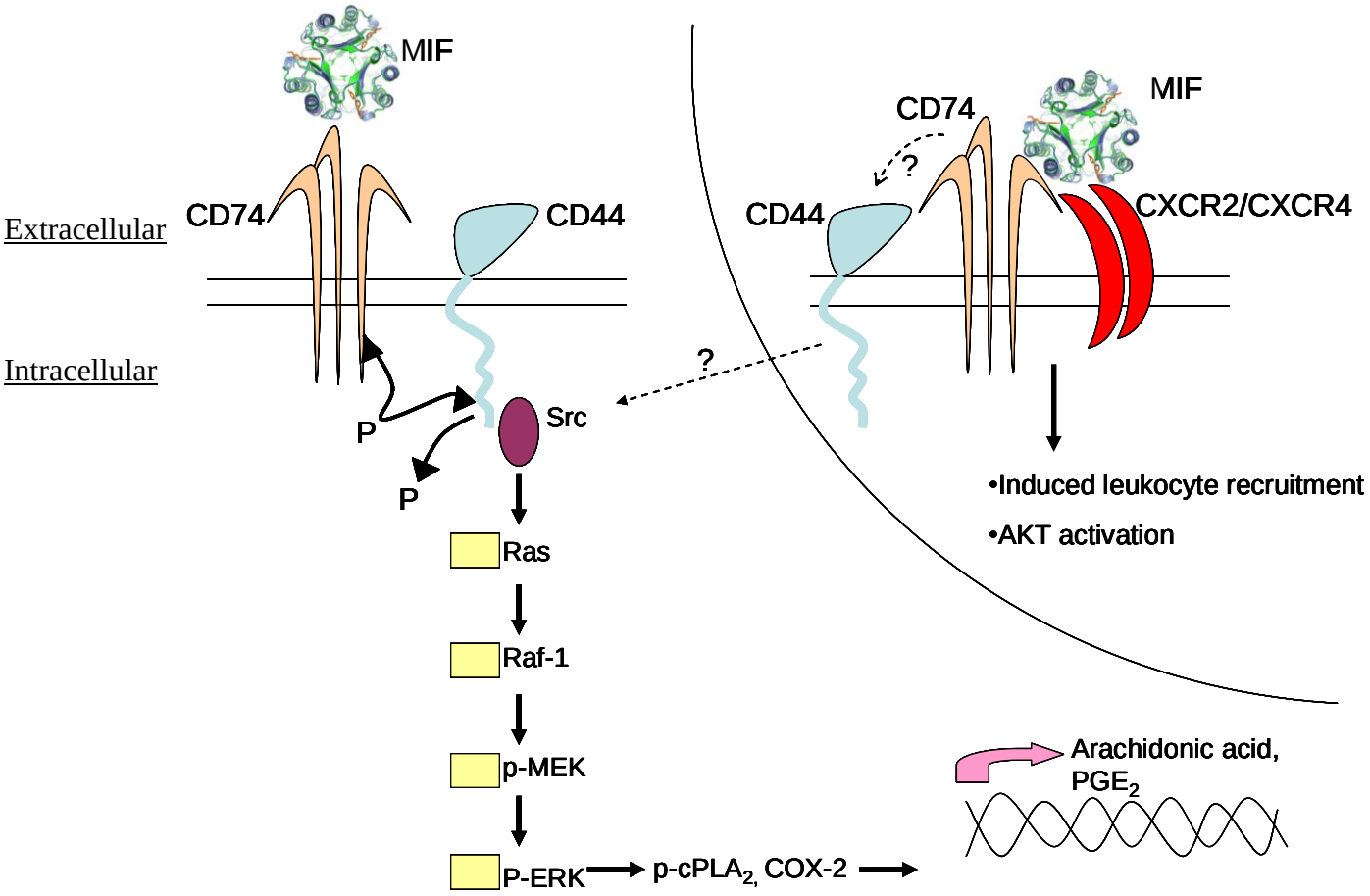


Figure 1.7 Proposed model of MIF signal transduction

MIF binding to CD74 results in serine phosphorylation of CD74 and phosphorylation of CD44 leads to an association with Src kinases. This leads to downstream ERK MAPK activation which leads to production of mediators such as PGE_2 and arachidonic acid via $cPLA_2$ and COX-2. MIF has also been shown to bind to CXCR2/CXCR4 which is capable of forming an interaction with CD74. Formation of this complex has been associated with induced leukocyte recruitment as well as AKT activation. It is currently unclear whether an interaction is also formed with CD44. Diagram based on Shi *et al.,* (2006).

1.14 Biological Activities of MIF

Currently, a wide array of MIF mediated activities have been described. Herein, a selection of MIF biological activities is discussed in detail, which are represented diagrammatically in Figure 1.8 and the biological characteristics of MIF homologues are described in tables 1.1, 1.2, 1.3 and 1.4.

1.14.1 MIF inhibits p53-dependent apoptosis of macrophages

The ability of MIF to regulate innate immune responses makes it a pivotal effector cytokine. Contrary to early studies that indicated T cells to be a major source of MIF (Bloom and Bennet, 1966, David 1966), it is now known that the activated macrophage is the predominant source of MIF within the immune system (Calandra *et al.,* 1994). Following activation with LPS, a series of mechanisms are initiated resulting in apoptosis, or programmed cell death (Albina *et al.,* 1993, Sarih *et al.,* 1993). Such mechanisms are the result of a regulatory process, which aims to keep innate immune responses in check that could otherwise result in immunopathological disorders such as septic shock and granulomatous disorders (Dinarello *et al.,* 2000). The overexpression of MIF has also been linked to these phenotypes, not unsurprisingly being a mediator of macrophage pro-inflammatory activity (Bernhagen *et al.,* 1993, Bozza *et al.,* 1999). Therefore, the apoptotic response is regarded as an important regulatory mechanism of the inflammatory response. The apoptotic process requires several mediators including the production of nitric oxide and the accumulation of tumour suppressor gene p53. Mitchell *et al.,* (2002) found that MIF regulated the activation pathway of the LPS induced apoptotic pathway of macrophages by inactivating p53 activity. These findings were complemented by an increase in arachidonic acid metabolism and a decrease in NO-induced p53 accumulation – this was not observed in $MIF^{-/-}$ macrophages. These findings therefore indicate a further role for MIF in maintaining macrophage pro-inflammatory function.

Species	Tautomerase	Oxidoreductase	Structure	Biological Characteristics	References
H. sapiens	• 3350μM/min/mg [1] 580μM/min/mg [2] L. dopa methyl ester tautomerase activity •Activity inhibited by ISO-1 and 4-IPP† •208μM/min/mg [3] p-hydroxyphenylpyruvate tautomerase activity	100m units [4] Reduction of 2-hydoxyethyldisulphide	Solved - Trimeric formation consisting of anti-parallel alpha helices packed against a four strand beta sheet [5]	•Inhibits random migration of monocytes/macrophages [6] •Inhibits p53 dependent apoptosis of macrophages [7] •Counteracts the anti-inflammatory effects of GC [8] • Induces lymphocyte activation [9] •Found in high concentrations in plasma of patients with severe sepsis [10] •Increases matrix metalloproteinases in RA [11] •Induces angiogenesis [12]	[1] Kamir *et al.*, 2008 [2] Tan *et al.*, 2001 [3] Cho *et al.*, 2007 [4] Kleemann *et al.*, 1998 [5] Sun *et al.*, 1996 [6] Hermanowski-vosatka *et al.*, 1999 [7] Mitchell *et al.*, 2002 [8] Calandra *et al.*, 1995 [9] Bacher *et al.*, 1996 [10] Calandra *et al.*, 2000 [11] Onodera *et al.*, 2000 [12] Shimizu *et al.*, 1999
M. musculus	•33.5 μmoles/min/mg L. dopa methyl ester tautomerase activity [13]	420m units[4] Reduction of 2-hydoxyethyldisulphide	Solved – Same trimeric architecture as HsMIF [14]	•Inhibits random migration of monocytes/macrophages [15,16] •Inhibits p53 dependent apoptosis of macrophages [7] •Upregulates TLR-4 expression [17] •Induces lymphocyte activation [9] •Induces ERK MAPK activation [18] •Inhibits NK cell activity [19] •Induces iNOS production in macrophages [19] •Promotes cell cycle progression in B cells [21] •Regulates insulin secretion [22]	[13] Richardson *et al.*, 2009 [4] Kleemann *et al.*, 1998 [14] Taylor *et al.*, 1999 [15] Bloom and Bennett, 1966 [16] David, 1966 [7] Mitchell *et al.*, 2002 [9] Bacher *et al.*, 1996 [17] Roger *et al.*, 2001 [18]Mitchell *et al.*, 1999 [19]Apte *et al.*, 1998 [20]Cuhna *et al.*, 1993 [21]Takahashi *et al.*, 1999 [22]Waeber *et al.*, 1997

Table 1.1 Enzymatic and biological characteristics of selected mammalian MIFs

Species	Tautomerase	Oxidoreductase	Structure	Biological Characteristics	References
A. americanum	•No data available	No data available, however CXXC motif is partially conserved.	Not solved	•Inhibits random migration of human MΦ equally as well as human MIF	Jaworski *et al.,* 2001
B. belcheri tsingtaunese	•Hydroxyphenylpyruvate tautomerase activity	Reduction of insulin by DTT but not GSH. CXXC motif is partially conserved.	Not solved	• No data available	Du *et al.,* 2006
H. longicornis	• No data available	No data available, however CXXC motif is partially conserved.	Not solved	•Inhibits random migration of human monocytes	Umemiya *et al.,* 2007
T. nigroviridis	•No data available	No data available, however a full CXXC motif is conserved	Not solved	•Inhibits random migration of macrophages by 35%	Jin *et al.,* 2007
X. laevis	•p-hydroxyphenylpyruvate tautomerase activity (equal to that of rat MIF)	No data available, however a full CXXC motif is conserved	Solved – Same trimeric architecture as HsMIF	•Is essential for axis formation and neural development of *Xenopus* embryos	Suzuki *et al.,* 2004

Table 1.2 Enzymatic and biological characteristics of MIF homologues from a selection of vertebrae and invertebrate species

Species	Tautomerase	Oxidoreductase	Structure	Biological Characteristics	References
A. ceylanicum	•150μmoles/min/mg p-hydroxyphenylpyruvate tautomerase activity •Activity unaffected by ISO-1	No data available. No conserved cysteines	Solved – Same trimeric architecture as HsMIF	•Binds CD74 • Inhibits random migration of human peripheral blood mononuclear cells	Cho *et al.*, 2007
B. malayi	•361.3μM/min/mg L. dopa methyl ester tautomerase activity [23] •106.8μM/min/mg p- hydroxyphenylpyruvate tautomerase activity [23]	No data available, however a full CXXC motif is conserved	Solved – Same trimeric architecture as HsMIF [23]	•Inhibits random migration of human monocytes/macrophages [24] •Increases recruitment of eosinophils [25] •Increases IL-8, TNF-α, endogenous MIF [23] •Synergises with IL-4 to induce alternative activation of macrophages [26]	[24] Pastrana *et al.*,1998 [2 5] Falcone *et al.*, 2001 [23] Zang *et al.*, 2002 [26] Prieto-Lafuente *et al.*, 2009
T. spiralis	•3500μmoles/min/mg L.dopa methyl ester tautomerase activity	No data available. No conserved cysteines	Solved – Same trimeric architecture as HsMIF	•Inhibits random migration of human peripheral blood mononuclear cells	Tan *et al.*, 2001

Table 1.3 Enzymatic and biological characteristics of MIF homologues from a selection of helminth species

Species	Tautomerase	Oxidoreductase	Structure	Biological Characteristics	References
E. tenella	•No data available, however, an N-terminal proline is present	No data available, however CXXC motif is partially conserved.	Not solved	• No data available	Miska *et al.*, 2007
L. major	•0.25mM/min/μM L. dopa methyl ester tautomerase activity [1] •Activity unaffected by ISO-1 but inhibited by 4-IPP[1] •1.2μmoles/min/mg L. dopa methyl ester tautomerase activity [13]	Small detectable oxidoreductase activity [13]	Solved – Same trimeric architecture as HsMIF[1,13]	•Inhibits random migration of human peripheral blood mononuclear cells •Induces ERK-MAPK activation[1] •Binds CD74[1] •Inhibits activation induced apoptosis of macrophages[1]	[1]Kamir *et al.*, 2008 [13]Richardson *et al.*, 2009
P. falciparum	•<20% activity of that in HsMIF p- hydroxyphenylpyruvate tautomerase activity	<30% activity of that in HsMIF C57 conserved HED reduced	Predicted structure indicates Same trimeric architecture as HsMIF	•Inhibits AP-1 activity in human embryonic kidney cells	Augustijn *et al.*, 2007

Table 1.4 Enzymatic and biological characteristics of MIF homologues from a selection of protozoan parasites

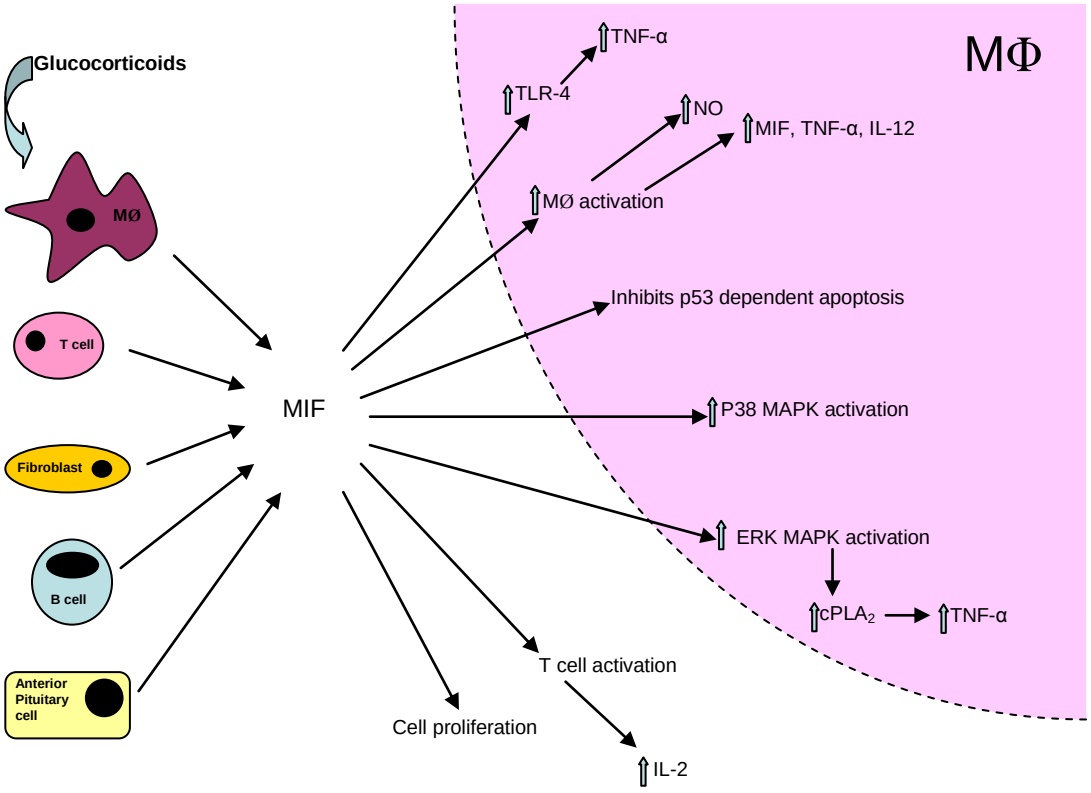


Figure 1.8 Biological activities of MIF

The diverse range of MIF biological activities are represented including MIF-mediated inhibition of p53 apoptosis of macrophages as well as activation of ERK-MAPK pathways. From this diagram, it is evident that the main target cell for MIF activity is the macrophage.

MIF mediated anti-apoptotic effects have also been observed in other studies. Kim *et al.,* (2008) demonstrated that patients with systemic sclerosis, a condition in which there is a dysregulated apoptosis of fibroblasts, had larger quantities of MIF present in their sera compared with healthy individuals. Furthermore, diseased fibroblasts were also more resistant to treatment with sodium nitroprusside (SNP), an apoptosis inducer, than those of healthy individuals. Furthermore, the inhibitory effect of MIF on apoptosis was ablated by the addition of an ERK inhibitor and protein kinase B inhibitor, indicating a role for these components in MIF-mediated protection from apoptosis (Kim *et al.,* 2008).

1.14.2 MIF counteracts immunosuppressive activity of glucocorticoids

MIF has also been implicated in regulating the anti-inflammatory effects of glucocorticoids (GC) (Calandra *et al.,* 1995). Initial observations by Bernhagen *et al.,* (1993) indicated that MIF was secreted from the same corticotrophic pituitary cells which secrete adrenocorticotrophin (ACTH), a mediator which results in the adrenal secretion of GC. This raised the idea that these mediators, MIF and ACTH, may interact having a consequence on immune target cells. Typically, GC stimulation results in a decrease in pro-inflammatory cytokine production. However, Calandra *et al.,* (1995) found that pre-incubating cells with increasing concentrations of MIF in the presence of the GC dexamethasone reduced dexamthasone's ability to inhibit pro-inflammatory cytokine production.

Indeed, pre-incubation of 1ng/ml MIF resulted in a ~60% reduction of dexamethasone mediated inhibition of TNF-α, IL-1β, IL-6 and IL-8. Similar results were obtained *in vivo* where MIF was shown to antagonise the protective effects of GC in lethal endotoxic shock. Furthermore, GC mediated downregulation of cell adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) was reversed following MIF stimulation, which results in increased leukocyte adhesion (Burger-Kentischer *et al.,* 2006, Amin *et al.,* 2006). In addition, GC regulate leukocyte turnover and trafficking in response to mild, acute stress. Rodents treated with anti-MIF antibody were found to enhance the stress-GC induced egress of peripheral leukocytes from circulation, consistent with the effects of GC action alone (Fingerle-Rowson *et al.,* 2003).

Surprisingly, stimulation of murine monocytes/macrophages with physiological concentrations of GC results in MIF secretion. However, high GC concentrations ($>10^{-8}$M) are able to prevent

MIF secretion. This indicates a tightly regulated system for MIF mediated regulation of GC activity (Calandra *et al.*, 1995).

The exact molecular mechanisms by which MIF and GC counter regulate each others activity is unknown. However, this activity has been observed in several biological systems. For example, MIF induced activation of ERK1/2 MAPK leads to phosphorylation of proteins and activation of transcription factors including cytosolic phospholipase A_2 ($cPLA_2$) which results in arachidonic acid production. Subsequently arachidonic acid activates jun-N-terminal kinase (JNK) which is required for TNF-α production (Mitchell *et al.*, 1999, Swantek *et al.*, 1997). However, GC inhibits $cPLA_2$ via a pathway involving annexin-1. Despite this, MIF continues to activate $cPLA_2$ even in the presence of GC (Flaster *et al.*, 2007).

P38 signalling pathways have also been shown to be involved in MIF modulation of GC activity on macrophages. TNF-α expression is regulated by the phosphorylation of MAPK activated protein kinase (Kotlyarov *et al.*, 1999). These kinases are in turn regulated by MAPK phosphatases (MKP) via dephosphorylation of MAPK whereby cells lacking MKPs exhibit increased activation of p38 and JNK MAPK (Wu *et al.*, 2005). Subsequently, overexpression of MKPs results in reduced production of TNF-α, a product of p38 kinase activation (Shepherd *et al.*, 2004). Aeberli *et al.*, (2006) demonstrated that in the complete absence of endogenous MIF, macrophages increase expression of MKP-1 whilst phenotypically they exert an increased responsiveness to GC (Roger *et al.*, 2005). Consequently, these results suggest that MIF regulates the GC effect on LPS induced TNF-α production via the expression of MKP-1.

Overall the evidence would suggest that there is a MIF-GC dyad whereby these mediators act in concert to counter regulate each others activities (Flaster *et al.*, 2007). MIF has been shown to control the immunosuppressive effects of GC to prevent inhibition of essential pro-inflammatory responses required to resolve infection. Conversely, the immunopathology which can result from overexpression of MIF is counter regulated by GC.

1.14.3 MIF upregulates TLR-4 expression

Preliminary experiments with macrophages transfected with plasmid containing an antisense MIF mRNA, showed a muted response to heat killed gram negative bacteria *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, as measured by a decrease in TNF-α and

IL-6 (Roger *et al.*,2001). In contrast, an effective response was measured following stimulation with *S. aureus* and the yeast particle zymosan. As gram positive and gram negative bacteria stimulate TLR-2 and TLR-4 respectively, the previous observation therefore indicates that MIF contributes to TLR-4, but not TLR-2 signalling in the macrophage (Roger *et al.*, 2001).

Immune cell recognition of LPS involves LPS binding protein (LBP) which transfers LPS to a receptor complex of CD14 and TLR-4. TLR-4 is then capable of activating NFκB which can lead to transcription of pro-inflammatory cytokines such as TNF-α. Expression levels of CD14 are unchanged in antisense treated macrophages in comparison to control cells. However, TLR-4 is significantly decreased in antisense MIF treated macrophages with a complimentary decrease in TNF-α also being observed. These effects are reversed by the addition of MIF (Roger *et al.*, 2001). Furthermore, immunoneutralization of extracellular MIF results in a decrease in TLR-4 expression which consequently alters the pro-inflammatory phenotype of the macrophage (Roger *et al.*, 2003). Therefore MIF has been shown to upregulate TLR-4 expression thus increasing macrophage responsiveness to LPS which consequently strengthens the innate immune response following challenge by endotoxin or gram negative bacteria.

1.14.4 MIF activates ERK MAPK pathways

Observations that MIF could promote cell proliferation (Bacher *et al.*, 1996, Chesney *et al.*, 1999) led to subsequent studies to determine MIF impact on cell signalling pathways associated with proliferation. A study by Mitchell *et al.*, (1999) demonstrated the ability of both endogenous and exogenous MIF to stimulate proliferation of the fibroblast cell line NIH/3T3 which coincided with a sustained increase in p44/p42 ERK MAPK phosphorylation which was maintained up to 24hours post stimulation. Previous studies in PC-12 cells had shown that cell differentiation was associated with a similar pattern of sustained ERK MAPK activation (Wu *et al.*, 1996). This prompted speculation that MIF might be playing a similar role in stimulating similar pathways for cell differentiation. Furthermore, activation of the ERK MAPK activation by MIF, also resulted in an increase of phosphorylation and activation of $cPLA_2$, of which arachadonic acid is a downstream product. Therefore, MIF induced release of arachidonic acid subsequently results in production of mediators such as prostaglandins and leukotrienes as well as *c-jun* N terminal kinase activation for pro-inflammatory cytokine production (Mitchell *et al.*, 1999). Of note, anti-inflammatory glucocorticoid inhibition of

TNF-α production is mediated via inhibitory effects on *c-jun* N terminal kinase pathway (Swantek *et al.*, 1997).

Further studies have also suggested a role for MIF in driving the phenotype of ectopic endometrial cells. MIF was found to upregulate cyclooxygenase 2 (COX-2) expression in endometrial cells which then resulted in an increase in prostaglandin E2 (PGE2) production (Carli *et al.*, 2009). Production of these mediators, coincided with an increase in activation of p38 and ERK MAPK pathways, suggesting MIF regulates production of COX-2 and PGE2 via these signalling pathways.

1.14.5 MIF activates T cells

In addition to innate immunity, MIF also drives adaptive immune responses, in particular T cell immunity. Bacher *et al.,* (1996) first discovered that activated T cells had increased levels of MIF, both at the mRNA and protein level. Furthermore, treatment with an anti-MIF antibody resulted in inhibition of T cell proliferation as well as a notable decrease in IL-2 production, a cytokine essential for the initiation of an effective T cell response. Therefore, from this study the authors were able to describe a role for MIF as an integral part of mitogenesis and subsequently T cell activation (Bacher *et al.*, 1996). However, contrary to this observation, others have dismissed a critical role for MIF in T cell activation as MIF deficient T cells have been found to proliferate as efficiently as wild type T cells in response to experimental parasitic infection (Rodriguez-Sosa *et al.*, 2003, Satoskar *et al.*, 2001).

1.15 MIF enzymatic activities

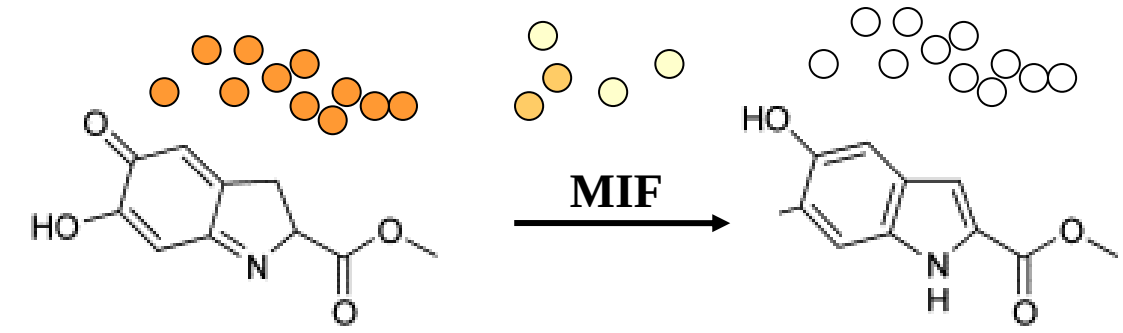
1.15.1 Tautomerase activity

In the early 1990's, MIF tautomerase activity was identified by Rosengren and colleagues (Figure 1.9). Prior to this investigation, a separate study investigating the later stages of melanin biosynthesis whereby the naturally occurring L- 2-carboxy – 2,3-dihydroindole-5,6-quinone stereoisomer (dopachrome) is converted to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Pawelek *et al.*, 1991) via a tautomerisation reaction which had been carried out in melanoma cells (Odh *et al.*, 1993). Curiously, in this study a tautomerase activity was measured from control melanoma cells which had been incubated with control substrate D-dopachrome. Subsequent investigations led to the identification of an enzyme called D-dopachrome tautomerase which was then identified in tissues including rat liver (Zhang *et al.*, 1995). Subsequently, Rosengren *et al.,* (1996) characterised a second enzyme capable of

driving this D-dopachrome tautomerase activity from bovine eye lens with subsequent isolation of the catalytic mediator being revealed as the bovine homologue of MIF. As a result, this group were first to firmly characterise MIF tautomerase activity and consequently human D-dopachrome tautomerase was cloned and expressed (Nishihira *et al.,* 1998). However, although MIF tautomerised dopachrome, it was evident that this was not the physiological substrate and so the search for this mediator begun. Since then, MIF has also been shown to catalyse phenylpyruvate and p-hydroxyphenylpyruvate tautomerase reactions (Rosengren *et al.,* 1997). However enzyme kinetic analysis shows that the Km for MIF with these molecules is 1000 times higher than the serum concentrations of these molecules which probably rules out these substrates as natural ligands (Deutsch, 1997).

At present, the natural *in vivo* substrate for MIF is unknown, however, an *in vitro* study has identified quinone products of dopamine and norepinephrine as potential natural substrates of MIF tautomerase activity, as they are readily converted to indole derivatives. Indole derivatives are precursors of neuromelanin and subsequently a role for MIF in neuromelanin synthesis has been suggested. However, despite this, there is currently a lack of consensus with regard to identification of the natural substrate. Indeed, if the natural substrate is structurally similar to dopachrome it is intriguing as to why MIF accepts the D-isomer of dopachrome and not the naturally occurring L-isomer (Rosengren *et al.,*1996). It has been suggested that such discrimination may be perhaps to avoid interference with melanin biosynthetic pathways (Bendrat *et al.,* 1997). However, MIF has been shown to catalyse L-dopachrome methyl ester (i.e. L-dopachrome with a modified carboxy group). Furthermore, the ability of MIF to tautomerise several neuropeptides has prompted suggestion that substrates bearing an N-terminal tyrosine may be natural MIF substrates (Bendrat *et al.,* 1997).

Elucidation of rat and human MIF structures revealed a surprising similarity with these proteins and the *E. coli* enzyme CHMI (Hajipour *et al.,* 1993, Sugimoto *et al.,* 1996) and 4-oxalocrotonate tautomerase (4-OT) (Bendrat *et al.,*1997). The catalytic base of CHMI and 4-OT is an N-terminal proline which is greatly conserved in *E.coli* (Subramanya *et al.,* 1996). The structural homology observed between CHMI and MIF, led to the suggestion that the catalytic site for MIF tautomerase activity may be focused around the N-terminal proline. Studies with 4-OT showed that incubation with the irreversible inhibitor 3-bromopyruvate (3-BP) resulted in a loss of activity by modification of the N-terminal proline. Subsequent inhibition studies with MIF and 3-BP also resulted in an irreversible complete loss of activity,



D,L-dopachrome methyl esters (orange)

Indole derivative (colourless)

Figure 1.9 MIF tautomerase activity

L.DOPA methyl ester is tautomerised by MIF to form 5,6-dihydroxyindole-2-carboxylic acid in a tautomerisation reaction which goes from orange to colourless. Specifically, tautomerisation of L.DOPA methyl ester involves the abstraction and migration of a proton, which is associated with the movement of a double bond.

suggesting an essential role for the MIF N-terminal proline (Bendrat *et al.*,1997, Stamps *et al.*, 1998). Consequently, it was shown that mutation of the N-terminal proline to an alanine resulted in a substantial decrease in human MIF tautomerase activity (Nishihira *et al.*,1998).

Early studies made no direct connections between MIF catalytic activity and immunological function. However, from the late 90's onwards, characterisation of the potential physiological role of MIF tautomerase activity was investigated in many studies. Bendrat *et al.*,(1997) reported that enzymatically inactive MIF retained its ability to counteract the anti-inflammatory effects of glucocorticoids. This result was later confirmed by Kleemann *et al.*, (2000) who observed that a series of N-terminally truncated mutants retained their full glucocorticoid overriding activity. In addition, Hermanowski-Vosatka *et al.*, (1999) also reported catalytically inactive mutants of MIF were still able to inhibit monocyte chemotaxis. However, these results are in contrast to Swope *et al.*, (1998) who demonstrated 'P1G' mutants (lacking the n-terminal proline) were catalytically inactive and displayed a reduced cytokine activity. Furthermore, contrary to previous results, Lubetsky *et al.*, (2002) found that 'PAM' mutants, (whereby an alanine is inserted between Pro-1 and Met2), failed to override the anti inflammatory effects of glucocorticoids. Such contrasting results have led some authors to suggest the importance of the catalytic site, but not necessarily enzyme activity in MIF immunological function (Dios *et al.*, 2002).

Despite being a somewhat contradictory and controversial area, many authors believe that the unique ability of MIF amongst cytokines to catalyse an enzyme reaction must indicate an immunological role for this enzymatic process. As such, many studies have researched the interactions of MIF with active site specific inhibitors, with a long-term goal for the development of new anti inflammatory agents. To date, the most commonly used inhibitor is (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1). Lubetsky *et al.*, (2002) investigated the ability of ISO-1 to inhibit MIF biological activity and found that the interaction of MIF with ISO-1 inhibited the ability of MIF to override GC anti-inflammatory activity. This was associated with a complementary decrease in TNF-α production from mononuclear cells as well as PGE_2 and COX-2. Al-Abed *et al.*, (2005) also reported that ISO-1 binding to the active site of MIF significantly inhibited the pro-inflammatory activity of MIF and as a consequence mice survival to severe sepsis was increased. Studies have also been carried out using alternative inhibitors. For example, Senter *et al.*, (2001) demonstrated that incubation of MIF with N-acetyl-p-benzoquinone imine

(NAPQI) resulted in an interaction with the catalytic site which inhibited the ability of MIF to inhibit glucocorticoid activity. Furthermore, Dios *et al.,* (2002) found that 2-[(4-hydroxybenzylidene)amino]-3-(1H-indol-3-yl)propionic acid methyl ester was a potent inhibitor of tautomerase activity which inhibited 3 aspects of MIF bioactivity: ERK MAPK activation, p53-dependent apoptosis of macrophages, and cell proliferation.

Understanding the mechanisms by which both substrates and inhibitors bind to the tautomerase active site will prove imperative to designing new inhibitors. For example, modelling studies show that ISO-1 and D-dopachrome interact in a similar manner with MIF, indicating Lys^{32} and Asn^{97} are two of the most important residues in MIF mediated activity (Lubetsky *et al.,*2002). Indeed many dopachrome analogs and pharmacological inhibitors have already been designed in the hope of better understanding MIF biological activity (Zhang and Bucala, 1999, Dios *et al.,* 2002).

1.15.2 Oxidoreductase activity

Kleemann *et al.,* (1998) described a role for human MIF as a thiol protein oxidoreductase (Figure 1.10). Initial analysis of the human MIF amino acid sequence highlighted a CXXC motif spanning between residues 57→60, with such motifs being the catalytic centres for many thiol protein oxidoreductases including thioredoxin (Trx) and glutaredoxin. Such observations led to the hypothesis that MIF exhibited a cysteine-dependent thiol protein oxidoreductase which was associated with the formation of an intramolecular disulphide bridge between two cysteines (Kleemann *et al.,* 1998). MIF was shown to be able to reduce both insulin and 2-hydroxyethyldisulphide (HED) via its oxidoreductase activity. However, MIF reduction of insulin was only observed when reduced glutathione was the applied substrate, an effect which was not observed in the presence of dithiothreitol (DTT) as the substrate.

In order to determine the importance of each individual cysteine in driving the oxidoreductase reaction, C57S and C60S mutants were created whereby the corresponding cysteines were substituted for serines (Kleemann *et al.,* 1998). Assays using these mutant proteins revealed that while C57S mutants had a reduced oxidoreductase activity, this activity was completely absent from C60S mutants. This data therefore indicated a more important role for Cys^{60} than Cys^{57} in mediating oxidoreductase activity. In addition, the authors also carried out some basic

immunological assays using the mutant proteins and found that the macrophage activating properties of MIF were inactive for C60S and significantly impaired for C57S mutants. These preliminary results indicate an immunological role for MIF oxidoreductase activity (Kleemann *et al.,* 1998). Later studies suggested a role for MIF oxidoreductase activity in cell redox homeostasis and MIF activation of monocytes and macrophages. However, these reports have not been further substantiated (Reviewed Thiele and Bernhagen, 2005)

In 2000, Kleemann *et al.,* performed the first comparative studies of the two enzyme activities and demonstrated that tautomerase activity was independent of the active site residues required for oxidoreductase activity and *vice versa*. Indeed, the only element that both activities are dependent on is the formation of the anti-parallel β1/β4 sheet in the monomeric MIF structure.

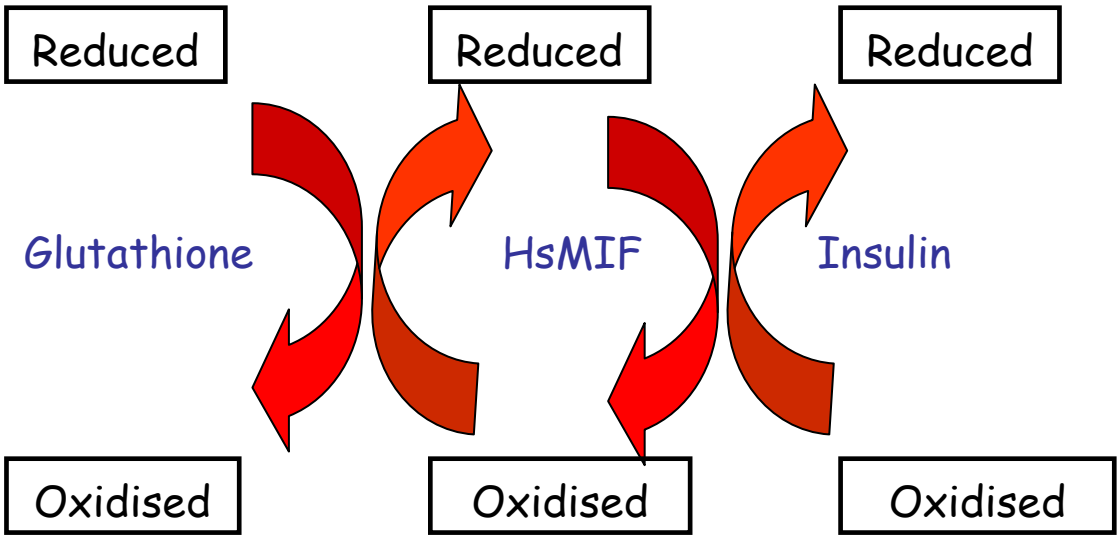


Figure 1.10 MIF oxidoreductase activity

The reduction of HsMIF by glutathione results in a break in the disulphide bridge found between Cys^{57} and Cys^{60} of HsMIF. HsMIF then reduces insulin into its subunit components whilst itself returning to its oxidised state. This is associated with the re-formation of the disulphide bridge between Cys^{57} and Cys^{60} of HsMIF.

1.16 MIF in infection and disease

Many studies have implicated MIF in having a major role during a variety of different infections and diseases. Outlined below is the role of MIF in selected infections and diseases.

1.16.1 MIF in septic shock

Severe sepsis initiated by gram positive and gram negative bacteria can be a major cause of death of hospitalised patients (Morrison and Ryan, 1987). Therefore, identification of the mediators responsible for this condition may help in the development of new therapeutics for preventing severe sepsis. A role for MIF in severe sepsis is well documented. Indeed, studies have shown that MIF is released from tissues such as lung, liver and kidney upon stimulation with LPS (Bacher *et al.,* 1997). Furthermore, a protective effect is observed in wild type mice upon administration of anti-MIF antibodies (Calandra *et al.,* 2000). In addition, $MIF^{-/-}$ mice challenged with 25mg/kg LPS to induce sepsis are more resistant to lethal endotoxaemia as evident by a 50% reduction of TNF-α in the plasma. This *in vivo* result is consistent with a reduction of TNF-α produced from murine $MIF^{-/-}$ macrophages, although IL-6 production is unaffected both *in vivo* and *in vitro* (Bozza *et al.,* 1999). The importance of both MIF and TNF-α in sepsis has been documented in several studies (Heumann *et al.,* 1995, Pfeffer *et al.,* 1993, Bozza *et al.,* 1999, Calandra *et al.,* 2000), which has prompted the suggestion that a potent interaction between these mediators is involved in determining the outcome of severe sepsis and septic shock.

MIF has been identified in high concentrations in the plasma of patients with severe sepsis or septic shock (Calandra *et al.,* 2000, Lehmann *et al.,* 2001). In particular, high levels of MIF in acute sepsis have been associated with a poor outcome in infection induced sepsis (Bozza *et al.,* 2004, Chuang *et al.,* 2007). Specifically, patients with septic shock had media plasma MIF levels of 17.8ng/ml whereas healthy individuals' plasma MIF levels were 3ng/ml. Patients who survive septic shock had 10.2ng/ml MIF in the plasma whereas non survivors 18.4ng/ml (Calandra *et al.,* 2000).

Inhibitors designed to specifically block the tautomerase active site of MIF have been shown to reduce disease severity and increase positive disease outcome to severe sepsis (Al-Abed *et al.,* 2005, Dabideen *et al,,* 2007). Alternatively, the development of a DNA MIF/tetanus toxin vaccine administered prophylactically can significantly reduce septic shock lethality (Tohyama *et al.,* 2008). Furthermore, a role for MIF in a non-LPS induced system of sepsis has been

described. MIF has been implicated in the pathogenesis associated with gram positive toxic shock syndrome (TSS) as TSS toxin-1 (TSST-1) induces production of MIF from macrophages. Prophylactic administration of anti MIF antibodies to C57BL/6 mice can increase survival from 8% to 54% following infection with TSST-1 (Calandra *et al.*, 1998)

1.16.2 MIF in arthritic conditions

MIF has been implicated as a constitutive factor in arthritic conditions with earliest studies successfully measuring MIF activity in synovial fluids and rheumatoid pericarditis (Geiger and Vischer, 1976, Lohrmann *et al.,* 1979, Andreis and Hurd, 1977). Furthermore, Mikulowska *et al.,* (1997) first reported a role for MIF in an animal model of rheumatoid arthritis (RA). They showed that treatment with neutralizing anti-MIF antibodies in murine collagen induced arthritis (CIA) resulted in a delayed onset of a milder form of this arthritic disease. These results were further complemented by work which demonstrated that CIA was suppressed in $MIF^{-/-}$ mice (Ichiyama *et al.,* 2004). Studies involving human participants have shown that MIF is overexpressed in cells and tissues in those individuals suffering from arthritis (Leech *et al.,* 1999). This increase in MIF in the joints is associated with increased severity of RA (Morand *et al.,* 2002) which in turn leads to an increase in matrix metalloproteinases which are responsible for the pathological features in RA (Onodera *et al.,* 2000, Pakozdi *et al.,* 2006). Furthermore, MIF has been shown to be involved in synoviocyte activation which leads to upregulation of pro-inflammatory mediators such as $cPLA_2$, COX2, IL-6 and IL-8 (Sampey *et al.,* 2001, Onodera *et al.,* 2004, Santos *et al.,* 2004). Finally, many studies have attempted to identify MIF gene polymorphisms associated with susceptibility to RA. Martinez *et al.,* (2007) for example, reported a polymorphism in the MIF promoter region that is associated with an increased predisposition to RA. With such an apparently prominent role for MIF in RA being described in the literature, small molecule inhibitors of MIF are currently being developed as therapeutic agents against this inflammatory condition (Reviewed Morand *et al.,* 2006).

1.16.3 MIF in cancer

The ability of MIF to promote cell growth led to studies investigating a potential role for MIF in tumorigenesis. Subsequently, an increase in MIF mRNA has been associated with many cancers of different tissues including liver, lung, colon and prostate with some cancers coinciding with an increase in disease severity with increased MIF mRNA levels (Akbar *et al.,* 2001, Kamimura *et al.,* 2000, Shkolnik *et al.,* 1987, Meyer-Siegler *et al.,* 1996). Indeed, many

of the biological activities of MIF are favourable for the development and maintenance of tumours. MIF induced inhibition of p53-dependent apoptosis has led to suggestion that MIF may enhance tumour cell viability which could lead to disease progression (Mitchell, 2004). Furthermore, MIF has been shown to interact with NM23-H1, a metastasis suppressor, directly via an interaction between MIF Cys^{60} and NM23-H1 Cys^{145}. This interaction was shown to alleviate MIF mediated suppression of p53 apoptosis of cells. Indeed, NM23-H1 was shown to inhibit MIF induced apoptosis of NIH 3T3 fibroblast cells with a corresponding decrease in MIF ERK MAPK activation also being observed. These results would therefore suggest a role for NM23-H1 as a negative regulator of MIF (Jung *et al.*, 2008).

Other examples of roles for MIF in cancers have been described. For example, MIF is capable of inducing angiogenesis and also promoting cell cycle progression, both of which are capable of influencing tumour growth and development (Mitchell and Bucala, 2000, Shimizu *et al.*, 1999, Takahashi *et al.*, 1999). Furthermore, there are several examples of specific cancers and correlations between MIF and tumour growth, maintenance and development. For example, sustained interaction of CD44, part of the MIF receptor, with Rho kinase initiates a signalling cascade which results in tumour progression in breast cancer (Bourguignon *et al.*, 2003). These results are consistent with previous work which indicates a role for sustained CD44 activation in other cancers (Bourguignon *et al.*, 2008, Bourguignon *et al.*, 2007, Wang *et al.*, 2007).

1.17 MIF in parasitic infections

Roles for MIF have been described in bacterial and viral infections and are reviewed elsewhere (Koebernick *et al.*, 2002, Bacher *et al.*, 2002). Herein the role of MIF in several parasitic infections are discussed.

1.17.1 MIF in *Leishmania* infection

MIF has been shown to play an important role in the immune response mounted following infection with *Leishmania*. Preliminary studies where recombinant human MIF was used to stimulate macrophages infected with *L. donovani* led to activation of macrophage anti-microbial pathways which suppressed parasitic growth and induced parasite killing. Indeed, in this study a reduction of parasites by ~50-77% was recorded post incubation with rHsMIF for 48 to 72 hours. This activation was found to be amplified by LPS and unaffected by IL-4 treatment (Weiser *et al.*, 1991). Subsequent studies showed that the delivery of human MIF,

either alone or in combination with TNF-α and IFN-γ via a delivery system involving attenuated *S. typhimurium* to BALB/c mice resulted in a reduction in disease manifestations following infection with *L. major*. Parasite loads were reduced by a factor of 3 and iNOS expression was increased compared with controls. In addition, lesion development was significantly reduced in treated animals (Xu *et al.*, 1998). Gessner and colleagues (Juttner *et al*., 1998) also demonstrated that significantly increased levels of MIF were found in the lymph nodes of *L. major*-infected mice one week post infection. Subsequently, they showed MIF induction of macrophage killing of *L. major* to be via a pathway which is dependent on both endogenous TNF-α and iNOS. They also demonstrated that this pro-inflammatory MIF activity could be inhibited by IL-10, IL-13 and TGF-β.

Furthermore, additional studies in $MIF^{-/-}$ mice highlighted increased susceptibility to cutaneous *L. major* infection as represented by the formation of larger lesions and greater parasite burdens (Satoskar *et al.*, 2001). Peritoneal macrophages cultured from these mice showed a significant reduction of NO and super-oxide. However, an increase in IL-6 production compared to wild type mice was also observed (Satoskar *et al.*, 2001). MIF release from CD4+ T cells, in addition to other mediators such as IFN-γ and TNF-α, has been also shown to contribute towards parasite destruction (Kar *et al.*, 2004). These studies might have implications for understanding disease in humans as increased MIF levels have been reported in the serum of patients suffering from acute cutaneous leishmaniasis.

1.17.2 MIF in *Plasmodium* infection

A variety of roles for MIF in response to infection with the malarial parasite *Plasmodium* have been described although very often these differences can be accounted for in significant part due to the diversity of the disease manifestations and patient conditions in different studies. MIF has been shown to be released from macrophages following ingestion of *P. chabaudi* infected erythrocytes with an increase in MIF secretion correlating with worsening disease severity (Martiney *et al.*, 2000. Furthermore, MIF has been suggested as the unknown host derived factor which is involved in suppression of erythropoiesis (Martiney *et al.*, 2000). Nevertheless further studies found a decrease in MIF production during *P. falciparum* infection as measured in the peripheral plasma (De Mast *et al.*, 2008). Interestingly, a decrease in plasma MIF has also been reported to coincide with a lower number of circulating lymphocytes in experimental *P. falciparum* infection (De Mast *et al.*, 2008). However, in the placental plasma a significant increase in MIF is observed in humans infected with *P. falciparum* despite

a reduction in IL-12 (Chaiyaroj *et al.*, 2004). A study in children with non cerebral malaria also reported a decrease in circulating MIF to levels ~1-4ng/ml compared with a post mortem study of children with cerebral malaria which were found to have virtually no MIF at all present in the cerebral area (Clark *et al.*, 2003). Indeed, MIF has been associated with pathophysiological consequences of malaria including malaria anaemia, despite reports that MIF expression is decreased during infection. McDevitt *et al.*, (2006) reported that MIF synergized with IFN-γ and TNF-α which are known antagonists of hematopoeisis. Furthermore, they showed MIF inhibited erythroid differentiation and haemoglobin production whilst $MIF^{-/-}$ mice showed increased survival following *P. chabaudi* infection.

1.17.3 MIF in *Trypansoma* infection

At present, there are only a few published investigations that describe the role of MIF in *T. cruzi* infection. Reyes *et al.*, (2006), demonstrated that $MIF^{-/-}$ mice developed higher parasitaemia and lower levels of pro-inflammatory cytokines IL-12, IL-18, IL-1β and TNF-α and consequently reduced NK cell activation than control mice. These results therefore suggest a role for MIF in host resistance to *T. cruzi.* The authors also reported that adaptive immunity was unaffected in $MIF^{-/-}$ mice contrary to previous reports indicating that MIF was critical for adequate T cell activation and proliferation (Bacher *et al.*, 1996).

1.17.4 MIF in *Toxoplasma* infection

Igarashi *et al.*, (1979) first identified MIF in the supernatants from spleen cells in mice infected with *T. gondii*. Recently, Ferro *et al.*, (2008) investigated the effect of soluble *T. gondii* antigen (STAg) on MIF expression in human first trimester placenta. The authors showed an increase in MIF production from chorionic villious explants treated with STAg 24 hours post incubation. Throughout pregnancy, steroid hormones are steadily increased including cortisol, which has immunosuppressive effects which decreases immunity to infections such as *T. gondii*. However, the ability of MIF to override GC activity, such as cortisol, is well documented (Calandra *et al.*, 1995). Therefore, similar to what has been suggested for malarial parasites, MIF may be acting with other mediators such as IFN-γ to resolve *T. gondii* infection during the first trimester (Chaisavaneeyakorn *et al.*, 2002). However, at present the impact of this specific response involving upregulation of MIF has not been studied in terms of consequence for the unborn foetus. Furthermore, the authors also showed that in keeping with previous literature, MIF induced increased expression of ICAM-1 in the syncytiotrophoblast

and adhesion of THP-1 cells to villious explants (Burger-Kentischer *et al.,* 2006, Amin *et al.,* 2006).

Further studies have revealed that $MIF^{-/-}$ BALB/c mice have increased susceptibility to both RH and ME49 strains of *T. gondii* compared with wild type mice. This increased susceptibility is associated with greater tissue pathology, greater parasite burdens and mortality (Flores *et al.,* 2008). Furthermore, the production of IL-1β, IL-12, IL-18, TNF-α, IFN-γ and NO was significantly reduced in $MIF^{-/-}$ BALB/c mice, a finding which is consistent with previous studies that demonstrate that MIF promotes IL-12 and TNF-α production from macrophages (Calandra and Roger, 2003). Also, $MIF^{-/-}$ bone marrow DCs displayed lower transcripts for IL-2p19/p35/p40 in addition to IL-1β and TNF-α. Furthermore, compared to wild type controls, peritoneal exudate cells from ME49 infected $MIF^{-/-}$ mice were found to have reduced levels of TNF-αR, IFN-γR and TLR-4 resulting in an impairment of pro-inflammatory responses. A comparison of susceptible C57BL/6 with resistant BALB/c mice revealed significantly reduced mRNA levels of MIF in C57BL/6, an observation which was mirrored by MIF concentration in the plasma compared to BALB/c (Flores *et al.,* 2008).

Finally, a preliminary study of MIF activity in humans during *Toxoplasma* infection has been carried out. Flores *et al.,* (2008) showed that non-AIDS patients who suffered from toxoplasmic encephalitis had significantly reduced levels of MIF in the brain compared with patients who died from fungal infection of the brain.

1.18 Parasite MIF homologues

The MIF protein is highly conserved with many homologues known to exist in various parasites such as *Plasmodium* spp., *Ancylostoma* spp. and *Toxoplasma gondii* (Augustijn *et al.,* 2006, Cho *et al.,* 2007). Outlined below are the main characteristic features of selected parasites.

1.18.1 *Brugia malayi* MIF

In 1998, Pastrana *et al.,* described the first characterisation of a homologue of a human cytokine from a parasitic species. Characterisation of recombinant *B. malayi* MIF *(BmMIF)* revealed conservation of mammalian MIF functions including migration inhibitory and chemotactic activities on human monocytes/macrophages. In addition, BmMIF was shown in

later studies to induce eosinophil recruitment *in vivo* when injected into the peritoneal cavity of mice. This was associated with a corresponding increase in eosinophil chemotactic factor 1, or Ym-1, with previous studies demonstrating a role for Ym-1 in eosinophil recruitment both *in vivo* and *in vitro* (Falcone *et al.,* 2001, Owhashi *et al.,* 2000). These results would therefore suggest an important link between macrophage activation and eosinophil chemoattraction elicited by BmMIF in helminth-induced inflammatory responses. Interestingly, eosinophil recruitment is not an attribute associated with human MIF, although eosinophils do secrete human MIF (Falcone *et al.,* 2001).

Later studies identified a second homologue of BmMIF (BmMIF-2) in the *B. malayi* Expressed Sequence Tag (EST) project (Zang *et al.,* 2002). BmMIF and BmMIF-2 have 40% and 27% sequence identity with human MIF, respectively and both homologues show conservation of structure with HsMIF (Pastrana *et al.,* 1998, Zang *et al.,* 2002). Both homologues have been shown to have an active L.DOPA methyl ester tautomerase activity whilst immunologically they can drive IL-8, TNF-α and endogenous MIF from activated macrophages (Zang *et al.,* 2002). Therefore, *Brugia* MIFs elicit similar pro-inflammatory effects on macrophages to mammalian MIFs. However, chronic helminth infection is typically associated with a type-2 anti-inflammatory response in which alternatively activated macrophages are prominent (Loke *et al.,* 2000). In particular, BmMIFs were shown to up regulate the IL-4Rα on macrophages and enhance alternative activation of macrophages induced by IL-4 (Prieto-Lafuente *et al.,* 2009). This is in keeping with previous results in which an increase in Ym1 was observed following BmMIF administration into the murine peritoneal cavity (Falcone *et al.,* 2001). However, *in vivo* alternative activation of macrophages by BmMIFs was not observed with murine MIF. Murine MIF alternative activation of macrophages may therefore require additional components such as IL-4 produced as a result of the adaptive immune response (Prieto-Lafuente *et al.,* 2009). The authors therefore suggest that depending on the milieu composition MIF may mediate alternative macrophage activation.

Finally, despite the overwhelming evidence for MIF as a pro-inflammatory mediator, its involvement in the inactivation of Jab-1 suggests a contradictory anti-inflammatory role. It is proposed that high concentrations of human MIF may in fact block pro-inflammatory cytokine gene expression in a negative feedback mechanism and subsequently, the secretion of BmMIF homologues by the nematode is an attempt to contribute towards such an effect (Bucala, 2000, Zang *et al.,* 2002).

1.18.2 *Trichinella spiralis* MIF

A homologue of mammalian MIF has also been isolated from the nematode species *Trichinella spiralis* (Pennock *et al.*, 1998, Tan *et al.*, 2001). *T. spiralis* MIF (TsMIF) has been shown to exhibit L.DOPA methyl ester tautomerase activity, which is even greater than that of HsMIF and any other homologue which has been characterised to date (Tan *et al.*, 2001). Structural studies revealed TsMIF to have a conserved global topology similar to that of HsMIF. However, differences in amino acid residues in the active site results in a shortened hydrophobic pocket which facilitates tautomerisation of L.DOPA methyl ester exceptionally well but not p-hydroxyphenyl-pyruvate (Tan *et al.*, 2001). Immunologically, TsMIF exhibited an effect on monocyte migration whereby TsMIF increased the migration of monocytes towards the source by 69% compared with 42% with recombinant HsMIF.

1.18.3 *Eimeria tenella* MIF

A homologue of MIF in the apicomplexan *E. tenella* was recently characterised by Miska *et al.*, (2007). *E. tenella* MIF (EtMIF) has 53% amino acid sequence identity with HsMIF and modelling software predicts conservation of the MIF trimer architecture in this homologue. Highest levels of MIF transcripts were detected in merozoites with lower quantities in sporulated and unsporulated oocysts. MIF transcripts were undetectable in sporozoites (Miska *et al.*, 2007). Finally, phylogenetic analysis reveals that Apicomplexan MIF sequences have a relationship with MIF-like sequences identified from *Arabidopsis thaliana* (Miska *et al.*, 2007).

1.18.4 *Plasmodium falciparum* MIF

In recent years, a MIF homologue has been identified in *Plasmodium falciparum*, that is constitutively expressed throughout the parasite's life cycle (Augustijn *et al.*, 2006). The *P. falciparum* MIF homologue (PfMIF) has 29% identity and 39% similarity when compared with HsMIF and the structure is conserved with HsMIF. Of interest, Augustijn *et al.*, (2006) first noted that conserved residues consistent between both MIF proteins were most abundant around the active site for tautomerase activity and less so for oxidoreductase activity. Therefore, measurement of a strong tautomerase activity for p-hydroxyphenylpyruvate from C-terminally his tagged PfMIF was not unexpected. Unlike other parasite MIFs that have only low levels of oxidoreductase activity, PfMIF was found to possess approximately 20% of the oxidoreductase activity reported for HsMIF. This activity accounts for one of the few cases of a functional oxidoreductase with considerable activity in a parasitic homologue. The PfMIF is

immunologically active and is capable of inhibiting the random migration of monocytes whilst also reducing surface expression of TLR-2, TLR-4 and CD86 on stimulated macrophages (Cordery *et al.,* 2007). Interestingly, PfMIF chemotactic activity was observed at lower concentrations than observed with HsMIF (Shao *et al.,* 2008).

Construction of knock out parasites revealed PfMIF is not an essential protein for the parasite at any stage of its life cycle (Augustijn *et al.,* 2006). Furthermore, this study also revealed that PfMIF binds the same surface receptor as human MIF-CD74. Interestingly, initial findings indicate that PfMIF binds CD74 with greater affinity than HsMIF (Augustijn *et al.,* 2006). Furthermore, PfMIF is capable of physically binding Jab-1 and regulating gene expression by inhibiting the transcription factor activator protein-1 (AP-1), an activity that in HSMIF has been demonstrated to be dependent on the oxidoreductase region (Kleemann *et al.,* 2000). Of interest, PfMIF has recently been identified from the sera of malaria patients (Shao *et al.,* 2008).

It would seem counter productive for the parasite to produce a protein which could ultimately lead to its expulsion from the host. Despite being a potent pro-inflammatory mediator, human MIF has also been shown to interact in anti-inflammatory pathways either by engagement with Jab-1 or via a mechanism dependent on concentration. Therefore, Augustijn *et al.,* (2006) have suggested that the production of the MIF protein homologue by *P. falciparum* is an attempt by the parasite to modulate host immunity from an inflammatory to an anti-inflammatory state.

In addition, Waters and colleagues have also described the characterisation of *P. berghei* MIF (PbMIF) (Augustijn *et al.,* 2006). Expression of PbMIF peaks in the trophozoite stages and is indeed expressed throughout all stages of the life cycle. PbMIF shares great homology with PfMIF although, PbMIF catalyses tautomerase activity and oxidoreductase activity less well than PfMIF.

1.18.5 *Leishmania major* MIF

Completion of the *L. major* genome in 2005 revealed two genes which had substantial homology with human MIF (Ivens *et al.,* 2005). One of these homologues, Lm1740MIF, was produced and was shown to exhibit a tautomerase activity which was 13 fold lower than Human MIF for L.DOPA methyl ester which was unaffected by the MIF inhibitor ISO-1 (Kamir *et al.,* 2008). Later studies detail the second *L. major* MIF homologue to be completely

inactive of tautomerase activity (Richardson *et al.*, 2009). Lm1740MIF was shown to stimulate monocyte migration which was unaffected by treatment with ISO-1. Furthermore, Lm1740MIF binds CD74 with a 3 fold lower affinity than human MIF for CD74. Following interaction with CD74, Lm1740MIF was shown to affect host cell responses by activating ERK1/2 MAPK pathways whilst also having an inhibitory effect on host cell monocyte/macrophage apoptosis associated with a decrease in phosphorylated p53 following addition of apoptosis inducer SNP. It is suggested that such activity would contribute towards parasite survival within the macrophage, indicating Lm1740MIF is a virulence factor (Kamir *et al.*, 2008).

1.19 Objectives

The enzymatic and biological characteristics of mammalian MIF have been well characterised in the past 45 years. Subsequently, a role for MIF has been described in many infections and diseases (Heumann *et al.*, 1995, Ichiyama *et al.*, 2004, Akbar *et al.*, 2001). Furthermore, identification and characterisation of homologues of MIF in several parasitic species including *B. malayi*, *L. major* and *P. falciparum* has indicated potential immunomodulatory roles for these secreted proteins (Pastrana *et al.*, 1998, Kamir *et al.*, 2008, Augustijn *et al.*, 2007).

The ubiquitous protozoan parasite *T. gondii*, which is easily transmitted throughout a huge animal reservoir, has evolved many strategies for modulating and evading host immune responses including secretion of parasite-derived molecules such as profilin and cyclophilin 18 (Yarovinsky *et al.*, 2005, Ibrahim *et al.*, 2009). Therefore, the identification of MIF homologues in other Apicomplexan parasites prompted suggestion that a homologue of MIF may be found in the *T. gondii* genome, which may exhibit immune modulatory properties. Towards an understanding of the role of *T. gondii* MIF, the following aims were formulated:

- Clone, express and purify recombinant C-terminally His-tagged MIF
- Assess biochemical properties of TgMIF and where possible determine enzyme kinetics of tautomerase and oxidoreductase activities
- Determine TgMIF crystal structure
- Characterise the immunological activity of recombinant endotoxin-free TgMIF *in vitro*

Chapter 2

Cloning, expression and purification of TgMIF

2.0 Abstract

A single copy of the MIF gene is found within the *T. gondii* genome which has 25% and 23% identity with human and mouse MIF, respectively. TgMIF was amplified from tachyzoite cDNA RH strain and ligated into expression vetor pET21a. Soluble recombinant TgMIF was then expressed in a bacterial expression system before undergoing purification and an endotoxin removal procedure. Purified, endotoxin free protein was then used for the generation of mouse polyclonal antibodies which were then subsequently used to confirm the presence of secreted TgMIF from RH tachyzoites.

2.1. Introduction

The *T. gondii* genome encodes a single copy of a homologue of macrophage migration inhibitory factor (MIF) (Accession number DQ344450). MIF is expressed throughout a wide range of phyla including mammals. While the role(s) of this protein is not known in many phyla, in mammals it is known to function as a primarily pro-inflammatory cytokine secreted by many cells including monocytes, macrophages, T cells and eosinophils. Subsequently, MIF is associated with a diverse range of biological functions. As the name suggests, MIF was originally described according to its ability to inhibit the random migration of macrophages (Bloom and Bennett, 1966 and David, 1966). However, MIF is now known to induce ERK-MAPK activation (Mitchell *et al.*, 1999) which can lead to the downstream production of other pro-inflammatory mediators including TNF-α, IL-6 and IL-8. MIF homologues have also been identified in other and have subsequently been shown to modulate host immune responses in the course of infection. Consequently it was considered worthwhile to investigate a MIF homologue in *T. gondii* (TgMIF) which could potentially influence the development and maintenance of the host immune response and therefore the pathogenesis of infection.

In this chapter TgMIF is cloned and recombinant TgMIF is produced in a bacterial expression system. Production of recombinant TgMIF will facilitate later studies in this thesis.

2.2. Materials and Methods

2.2.1 Bioinformatical analysis

MIF sequences from VEG, ME49, GT-1 (*T. gondii* strains) and *Neospora caninum* were retrieved from the *T. gondii* genome project (http://toxodb.org). All other MIF sequences were acquired from NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez) with the exception of *P. falciparum* which was retrieved from the *Plasmodium* genome project (http://plasmodb.org). Sequence alignments were performed using Vector NTI (Invitrogen, Paisley, UK).

2.2.2 Maintenance of *Toxoplasma gondii* RH (Type-1) strain

T. gondii tachyzoites were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with L-glutamine and HEPES supplemented with 10% heat inactivated FCS, 100U/ml penicillin (Cambrex Bioscience, Veniers, Belgium), 100µg/ml streptomycin (Cambrex Bioscience, Veniers, Belgium) and 50U/ml amphotericin B (Cambrex Bioscience, Veniers, Belgium). RH tachyzoites were routinely maintained in human foreskin fibroblast (HFF) cells in 75cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland) in 10ml of the parasite culture media detailed above at 37°C and 5% CO_2. Tachyzoites were then harvested once ~80% parasites were intracellular. Parasites were harvested from infected cells with a 24cm cell scraper (TPP, Trasadingen, Switzerland) and diluted 10x in parasite culture medium and passed to a new confluent flask of HFFs.

2.2.3 RNA isolation

Toxoplasma tachyzoites, which had been maintained in HFF cells, were harvested from T75cm^2 tissue culture flasks for extraction of RNA. Typically, 10^8 parasites were harvested in 1ml Trizol. RNA was then isolated using the Chomczynski and Sacchi (1987) method of extraction. To expand, samples stored in Trizol (Invitrogen, Paisley, UK) were lysed by resuspension in a 25 gauge syringe prior to the addition of 0.5ml chloroform (Sigma-Aldrich) and centrifugation at 10,000g for 15 minutes at 4°C to separate the aqueous and organic phases. The aqueous layer was retained and the RNA was recovered by precipitation with 0.6ml isopropanol (Sigma-Aldrich). Samples were incubated for 10minutes at room temperature before centrifugation at 10,000g for 10minutes at 4°C after which the isopropanol (Sigma-Aldrich) was removed using a syringe being careful not to disturb the RNA pellet. At this point, 0.5ml Trizol (Invitrogen) was added to samples and centrifuged at 10,000g for 15minutes at 4°C. The process outlined above from retention of the aqueous layer was repeated after which the RNA pellet was washed in 1ml 70% ethanol (Sigma-Aldrich) before

centrifugation at 7000g for 15minutes at 4°C. The ethanol was removed and dried under a hood for 15minutes prior to the addition of 50µl molecular grade water (Invitrogen). RNA was solubilised at 65°C for 5 minutes. Samples were then quantified on a spectrophotometer, with typical yields of 2000-3000µg/ml being recorded.

2.2.4 Testing RNA for gDNA contamination

In order to ensure RNA samples were free from genomic DNA (gDNA) contamination, tata box binding protein (TBP) (192bp) was amplified by polymerase chain reaction (PCR) from each sample. PCR reactions containing 12.5µl 2x ReddyMix (1.5mM $MgCl_2$) (ABGene, Epsom, UK), 10.5µl molecular grade water (Sigma-Aldrich, Poole, UK) and 25pmol of each forward and reverse primer and 1µl RNA derived from RH strain mRNA. For negative controls, 1µl molecular grade water was added instead of cDNA. PCR reactions were performed using the following thermal profile: 1 cycle at 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, annealing temperature for 45 seconds, 70°C for 60 seconds. This was followed by a final elongation step of 70°C for 10 minutes. Samples were kept at 4°C until analysis by agarose gel electrophoresis when PCR products were loaded on to a 1.8% agarose gel, containing 0.00003% ethidium bromide (Sigma-Aldrich) and ran at 120 volts for approximately 50 minutes. PCR products were then visualised by UV transillumination (VWR GenoSmart, Lutterworth, UK) and bands of 192bp, representative of TBP, indicated gDNA contamination. Contaminated samples were treated with 1µl DNase I amplification grade (Invitrogen, Paisley, UK) for 1 hour at 37°C. Samples were treated with DNase and tested by PCR until RNA samples were deemed to be free of contamination.

2.2.5 cDNA synthesis

cDNA was synthesised from 2µg RNA in a reaction with 1µl random primers to which molecular grade water (Invitrogen) was added to a final volume of 14.2µl. Samples were then incubated at 65°C for 5 minutes and then slowly cooled to room temperature for 10 minutes to allow primers to anneal. To each sample, 2µl 10xAffinityScript Reverse Transcriptase buffer, 2µl 100mM DTT, 0.8µl 100mM dNTP mix and 1µl Affinity Script Multiple Temperature Reverse Transcriptase was added. Samples were then incubated at 25°C for 10minutes, 55°C for 1 hour and 70°C for 15 minutes. cDNA samples were then tested by PCR (see chapter 2.2.4) by amplification of TBP.

2.2.6 Amplification of TgMIF by Polymerase Chain Reaction (PCR) from RH cDNA

TgMIF was amplified by PCR reactions containing 12.5µl 2x ReddyMix (1.5mM $MgCl_2$) (ABGene, Epsom, UK), 10.5µl molecular grade water (Sigma-Aldrich, Poole, UK) and 25pmol of each forward and reverse primer and 1µl cDNA derived from RH strain mRNA. For negative controls, 1µl molecular grade water was added instead of cDNA. PCR reactions were performed using the following thermal profile: 1 cycle at 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, annealing temperature for 45 seconds, 70°C for 60 seconds. This was followed by a final elongation step of 70°C for 10 minutes. Samples were kept at 4°C until analysis by agarose gel electrophoresis when PCR products were loaded on to a 1.8% agarose gel, containing 0.00003% ethidium bromide (Sigma-Aldrich) and ran at 120 volts for approximately 50 minutes. PCR products were then visualised by UV transillumination (VWR GenoSmart).

2.2.7 Extraction of DNA from Agarose Gel

PCR products were excised from the agarose gel using a sharp scalpel and subsequently DNA was extracted using Qiagen Gel Extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instruction. The excised gel slice was weighed and dissolved by incubation at 50°C for 10 minutes in 3 volumes of Buffer QG per 1 gel volume. In order for nucleic acids to precipitate, 1gel volume of isopropanol was added to the column prior to centrifugation at 9400g for 1minute. The DNA was then washed in 750µl Buffer PE and subject to a final 'dry spin' before the DNA was eluted in 30µl Buffer TE. DNA was then quantified using a spectrophotometer.

2.2.8 Plasmid and PCR product restriction enzyme digest

In preparation for ligation, 10µl plasmid (pET21a)/PCR product (TgMIF) were incubated with1µl *Nde*I (Promega, Southampton, UK), 1µl *Xho*I (Promega), 2µl buffer D (Promega) in a final volume of 10µl at 37°C for 2 hours.

Following digest, PCR products were purified using Qiagen DNA clean up kit (Qiagen) according to the manufacturer's instructions. In brief, to the restriction enzyme digest reaction, 100µl Buffer PB was added before addition of the complete reaction mixture to a MinElute spin column. Columns were then washed with 750µl Buffer PE. Finally, the column was spun

dry for 1 minute before the DNA was eluted into a clean microcentrifuge tube in 10µl Buffer EB.

Following digest, linearised plasmid (pET21a) was run on an agarose gel and the plasmid band was excised and subject to purification by MinElute Gel Extraction kit (Qiagen) as described above.

2.2.9 Ligation

The excised DNA fragment, TgMIF, was ligated into a cloning vector, pET21a (Novagen). In a 10µl reaction, 3µl of TgMIF PCR product was added to 5µl pET21a (plasmid), 1µl T_4 ligase (Invitrogen) and 1µl T_4 ligase buffer (Invitrogen). The ligation mixture was incubated at 4˚C overnight.

2.2.10 Competent Cell Preparation

A colony of DH5α/BL21 cells were selected from an agar plate and inoculated in 10ml of LB broth (USB corporation. Cleveland, USA) which was subsequently incubated at 37ºC on a rotary shaker (225rpm) overnight. 5ml of the bacterial broth was transferred aseptically to 100ml of LB broth and incubated at 37ºC for 1-2 hours on a rotary shaker (225rpm) to a cell density of OD_{500} 0.5. The culture was then incubated on ice for 15 minutes, followed by centrifugation at 685g for 5 minutes. Subsequently, the supernatant was removed and the bacterial pellet was resuspended in 10ml of TFB1 (transformation buffer 1: 100mM RbCl, 50mM $MnCl_2$, 30mM KCl, 10mM $CaCl_2$, 15% glycerol, pH5.8). The culture was then placed on ice for 90 minutes which was proceeded by centrifugation of the bacterial culture at 685g for 5minutes. The supernatant was removed and the bacterial pellet resuspended in 2.8ml TFB2 (transformation buffer 2: 10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% glycerol, pH7) and incubated on ice for one hour. 100µl aliquots of the competent cells were placed into 1.5ml microcentrifuge tubes and stored at -70ºC.

2.2.11 Transformation of Competent Cells

In a transformation reaction, 2.5µl of ligation mixture was added to 50µl of BL21 chemically competent bacteria (Invitrogen) and incubated on ice for 20 minutes. Bacteria were then heat shocked by placing in a water bath at 42ºC for 2 minutes followed by a further 2 minute incubation on ice. 200µl of LB broth was added to the transformed culture which was then incubated at 37ºC for one hour on a rotary shaker (225rpm) (Cohen *et al.,* 1972).

Transformed cells were spread aseptically on an LB agar plate (USB corporation, Amsterdam, The Netherlands) which had been previously coated with ampicillin (USB corporation), final concentration 100µg/ml, and incubated at 37ºC overnight.

2.2.12 Colony screening

A colony was selected from the agar plate then added aseptically to LB broth containing 10µl ampicillin and incubated overnight at 37˚C on a rotary shaker (225rpm).

2.2.13 Plasmid purification

To purify the plasmid from the bacterial culture, three 1.5ml aliquots of the bacterial culture were centrifuged at 9400g for 1 minute, producing bacterial pellets. Purification was then carried out using QIAprep® spin Miniprep Kit (Qiagen). Briefly, pellets were resuspended in a total volume of 250µl resuspension Buffer P1 before the addition of 250µl lysis Buffer P2, to which 350µl neutralisation Buffer N3 was added. Samples were then centrifuged for 10 minutes at 9400g after which the supernatants were applied to a QIAprep Spin Column and spun again at 9400g for 1minute. The column was then washed by the addition of 750µl Buffer PE before a final elution of the plasmid DNA in 50µl Buffer EB into a clean microcentrifuge tube.

2.2.14 Screening by restriction enzyme digest

In order to ensure successful cloning of TgMIF into pET21a, 5µl purified plasmid was digested with 1µl *Nde*I, 1µl *Xho*I, 1µl Buffer D and 2µl molecular grade water and then incubated at 37°C for 2 hours. Digest products were visualised on a 1.8% agarose gel (see chapter 2.2.4).

Clones were sequenced by Geneservice (Cambridge, UK) to confirm successful amplification of the entire TgMIF gene.

2.2.15 SDS-PAGE

Preparation of one polyacrylamide gel comprised of a 14% resolving gel which consisted of 4.5ml deionised water, 2.8ml ProSieve 50 gel solution (Cambrex, UK), 2.5ml 1.5M Tris HCl pH 8.8, 100µl 10% SDS solution, 4µl TEMED (Sigma-Aldrich) and 0.1ml APS (Sigma-Aldrich). The gel was then added to a preassembled cassette (Invitrogen, Paisley, UK) on top of which a layer of water was also added. The gel was then allowed to solidify. Following solidification, the water layer was removed and 3mls stacking gel was added consisting of

2.25ml deionised water, 300µl ProSieve50 gel solution (Cambrex), 390µl 1M Tris HCl, pH6.8, 30µl 10% SDS solution, 3µl TEMED (Sigma) and 10µl APS (Sigma).

2.2.16 Small Scale Protein Expression

A colony of BL21 cells containing pET21a+TgMIF was picked from an agar plate and inoculated into a 10ml LB broth, containing 10µl ampicillin (100mg/ml), and incubated at 37ºC overnight with shaking at 225rpm. 1ml of the overnight culture was added to a fresh 10ml broth and incubated at 37ºC with shaking at 225rpm until an $O.D_{600}$ 0.6 was achieved. A 1ml pre-induced sample was taken. Protein expression was then induced with 1mM IPTG (ABgene) at 30ºC overnight. An uninduced control was also incubated. 1ml samples were taken following the overnight incubation which were subsequently centrifuged for one minute at 9400g. The supernatant was discarded and the pellet was resuspended in 50µl PBS pH7.4 to which a further 50µl sample buffer was added. Samples were boiled and then ran on a polyacrylamide gel at 120 volts for 1 hour and 30 minutes. Following this, the gel was then separated from the cassette and placed in 40mls Coomassie® blue overnight, after which, the gel was destained in destaining buffer (10% acetic acid, 10% methanol) until bands were visible.

2.2.17 Solubility

Protein expression was induced as detailed previously. Induced broths were then centrifuged for 5 minutes at 6000g and pellets were resuspended in 5mls PBS containing protease inhibitors (Roche biochemicals, Lewisham, UK). Broths were sonicated using Sonics Vibra Cell at 60kHZ. Samples were sonicated on ice for 6 x 10 second pulses with intermittent breaks followed by centrifugation for 5 minutes at 6000g and 4ºC. The pellet was then resuspended in 5mls of PBS and protease inhibitors (Roche Biochemicals), following separation from the supernatant. The resuspended pellet and supernatant were then frozen overnight at -20˚C. Once more, the resuspended pellet and supernatant were sonicated for 12 x 10 second pulses with intermittent breaks followed by centrifugation for 5 minutes at 6000g at 4ºC and the new pellets were then resuspended in 5mls of PBS and protease inhibitors (Roche Biochemicals), following separation from the supernatant. Samples from the sanitation procedure were run on a polyacrylamide gel alongside a protein marker (Promega) at 120 volts for 1 hour and 30 minutes.

2.2.18 MALDI-TOF mass spectrometry

Bands thought to be representative of TgMIF were excised from acrylamide gel and sent to the proteomics facility at the University of Glasgow for MALDI-TOF mass spectrometry.

2.2.19 Large Scale Protein Expression and Purification

A colony of BL21 cells containing pET21a+TgMIF was selected from an agar plate and inoculated into a 10ml LB broth and incubated at 37ºC overnight. 5ml of the overnight culture was added to a fresh 1L broth and incubated at 37ºC until an $O.D_{600}$ 0.6 was achieved. Protein expression was then induced with 1mM IPTG (ABgene) at 30ºC overnight. The cells were harvested by centrifugation at 6000g for 20minutes and the resulting pellet was resuspended in 20ml lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH8) prior to incubation with 100µl lysozyme (1mg/ml) at 4°C for 30mins. The lysate was then sonicated for 6 x 30 seconds with equal intermittent breaks followed by centrifugation at 6000g for 30 minutes.

2.2.20 Protein purification

A polypropylene column was packed with 1ml Ni-NTA agarose and allowed to solidify before equilibration with 10ml lysis buffer. Following equilibration, supernatant from the previous step was added and allowed to pass through the column by gravity flow. Flow through samples were collected for analysis by SDS PAGE. Once the lysate had passed through the resin, the column was washed with 30ml wash buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH8). Finally, purified protein was eluted in 100µl fractions in elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM imidazole, pH8).

2.2.21 Protein Estimation by bicinchoninic acid (BCA) assay

Protein concentration was quantified using BCA assay (Pierce Rockford, UK). To a 96 well plate, 10µl standard (2→ 0.025mg/ml) and 10µl sample were added in duplicate to appropriate wells. To each well, 200µl working reagent(Reagent B: Reagent A 1:50) was added before the plate was covered in tin foil and incubated at 37°C for 30minutes. The plate was then read at 562nm on a SpectraMax (Molecular Devices, Sunnyvale, USA).

2.2.22 Western Blot Analysis

Proteins were transferred from a polyacrylamide gel (described above) to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) using the Novex Xcell Blot Module (Invitrogen) at 30volts for 90 minutes. The membrane was blocked for 2 hours with 20ml

0.2% BSA in NATT buffer (50mM Tris-HCl, 150mM NaCl, 0.2% (v:v) Tween 20) after which the membrane was incubated overnight at 4°C with an anti his antibody (1:1000 dilution) (Cell Signalling Technology, Massachusetts, USA). The following morning, the membrane was washed with NATT buffer for 90minutes, changing the wash buffer every 15 minutes. Anti-rabbit IgG HRP-linked secondary antibody (Cell Signalling Technology, Massachusetts, USA) was diluted 1:1000 in 0.2% BSA in NATT and incubated with the membrane for two hours at room temperature. Following this, the membrane was washed with NATT buffer for 90minutes, changing the wash buffer every 15 minutes. Antibody binding was measured by chemiluminescence and the incubation of 2ml ECL substrate (Pierce) for two minutes before the membrane was pressed in a cassette for 1 minute. The film was then developed by a Kodak M-35-M- X-OMAT processor.

2.2.23 Generation of polyclonal anti-TgMIF antibodies

In order to generate polyclonal antibodies, 5 female BALB/c mice were injected subcutaneously into the rump with 100μg TgMIF absorbed in ALUM. Two weeks following injection, tail bleeds were taken to confirm the presence of anti TgMIF antibodies. Four weeks post-injection blood samples were collected by cardiac puncture and plasma was collected by centrifugation of samples at 9400g for 5 minutes.

2.2.24 Preparation of *T. gondii* supernatants

The media from a flask of HFF's infected with *T. gondii* RH tachyzoites was collected and concentrated using 5000MWCO Vivaspin columns (Sartorius, Goettingen, Germany). Concentrated protein was collected following a single centrifugation step and quantified by BCA assay. Samples were prepared for SDS-PAGE by the addition of sample buffer (0.5M Tris HCl, 20% Glycerol, 5% 2-mercaptoethanol, 5% (0.1% w/v) bromophenol blue, 40% (10% w/v) SDS) followed by boiling to denature the proteins. 5μg was added to each well of an polyacrylamide gel.

2.2.25 Preparation of *T. gondii* Excretory/Secretory Antigen (ESAg)

T. gondii RH tachyzoites were adjusted to $1x10^7$ parasites/ml and incubated in sterile PBS and 1% FCS for 3 hours (Henriquez *et al.*, 2005). Following incubation, parasites were centrifuged for 15minutes at 9400g after which the supernatant was removed and centrifuged for a second time for 15minutes at 9400g. The supernatant was recovered and quantified by the BCA assay.

Samples were prepared for SDS-PAGE by the addition of 2xsample buffer, followed by boiling to denature the proteins. 5μg was added to each well of an acrylamide gel.

2.2.26 Anti-TgMIF Western Blot

Proteins were transferred from a polyacrylamide gel (described above) to a nitrocellulose membrane (Amersham Biosciences) using the Novex Xcell blot Module (Invitrogen) at 30 volts for 90 minutes. The membrane was blocked for 2 hours with 20mls 0.2% BSA in NATT buffer (50mM Tris-HCl, 150mM NaCl, 0.2% (v:v) Tween 20) after which the membrane was incubated overnight at room temperature with an anti-TgMIF antibody (1:7500 dilution). The following morning, the membrane was washed with NATT buffer for 90minutes, changing the wash buffer every 15 minutes. Anti- mouse IgG HRP linked secondary antibody was diluted 1:10000 in 0.2% BSA in NATT and incubated with the membrane for two hours at room temperature. Following this, the membrane was washed with NATT buffer for 90minutes, changing the wash buffer every 15 minutes. Antibody binding was measured as previously described.

2.3 Results

2.3.1 Bioinformatical analysis of MIF homologues found in different strains of *T. gondii* and other Apicomplexa

Alignments of MIF genes from four strains of *T. gondii* (RH, GT1, ME49, VEG) were assembled and analysed using Vector NTI (Invitrogen) (Figure 2.1a). Sequence identity between all strains is 99.7% with the only difference in nucleotide sequence being observed at position 21. In GT1 strain, a thymine (T) is replaced with a cytosine (C). However, this substitution is not observed in any of the other strains analysed and does not result in an amino acid change.

Amino acid sequence alignments between Human MIF(HsMIF), Mouse MIF(MmMIF), TgMIF and other Apicomplexan MIFs (*P. falciparum, E. tenella* and *N. caninum*) were assembled and analysed using VectorNTI (Invitrogen) (Figure 2.1B). Sequence identity between TgMIF and HsMIF, MmMIF, PfMIF, EtMIF and NcMIF was 25.9%, 23.3%, 43%, 32.6% and 76.6% respectively (Figure 2.1B).

No classical von Heijne secretory signal has been identified in TgMIF. This is similar to other MIFs, where the lack of an N-terminal signal sequence for protein targeting to secretory organelles is evident.

2.3.2 Amplification of TgMIF from tachyzoite and bradyzoite cDNA

In order to determine the expression profile of TgMIF, the gene was amplified from tachyzoite and bradyzoite cDNA (Figure 2.2) by conventional RT-PCR using a standard PCR protocol at an annealing temperature of 64°C and PCR products were visualised on a 1.8% agarose gel containing ethidium bromide. A PCR product of 351bp was observed in the both stages.

2.3.3. Amplification of TgMIF from tachyzoite cDNA RH strain for ligation into expression vector pET21a

TgMIF was amplified from tachyzoite cDNA RH strain using forward primer 5`-GGGGCATATGCCCAAGTGCATGATCTTTTGCC-3` and reverse primer 5`-GGGGCTCGAGGCCGAAAGTTCGGTCGCCCATGGC-3` which were engineered to contain restriction enzyme sites *Nde*I and *Xho*I (underlined) and 5' and 3' ends respectively, suitable for ligation into expression vector pET21a (Invitrogen) (Figure 2.3a). PCR product

```
                1                                                  50
  GT1 MIF     (1) ATGCCCAAGTGCATGATCTTCTGCCCCGTCGCGGCGACGCCGGCGCAGCA
 ME49 MIF     (1) ATGCCCAAGTGCATGATCTTTTGCCCCGTCGCGGCGACGCCGGCGCAGCA
 RH TgMIF     (1) ATGCCCAAGTGCATGATCTTTTGCCCCGTCGCGGCGACGCCGGCGCAGCA
  VEG MIF     (1) ATGCCCAAGTGCATGATCTTTTGCCCCGTCGCGGCGACGCCGGCGCAGCA
Consensus     (1) ATGCCCAAGTGCATGATCTTTTGCCCCGTCGCGGCGACGCCGGCGCAGCA

                51                                                 100
  GT1 MIF    (51) GGACGCCCTCTTGAAGGACGCCGAAAAAGCCGTCGCAGACGCTCTGGGGA
 ME49 MIF    (51) GGACGCCCTCTTGAAGGACGCCGAAAAAGCCGTCGCAGACGCTCTGGGGA
 RH TgMIF    (51) GGACGCCCTCTTGAAGGACGCCGAAAAAGCCGTCGCAGACGCTCTGGGGA
  VEG MIF    (51) GGACGCCCTCTTGAAGGACGCCGAAAAAGCCGTCGCAGACGCTCTGGGGA
Consensus    (51) GGACGCCCTCTTGAAGGACGCCGAAAAAGCCGTCGCAGACGCTCTGGGGA

                101                                                150
  GT1 MIF   (101) AGCCTCTGAGCTACGTCATGGTGGGATACTCGCAGACCGGGCAGATGCGT
 ME49 MIF   (101) AGCCTCTGAGCTACGTCATGGTGGGATACTCGCAGACCGGGCAGATGCGT
 RH TgMIF   (101) AGCCTCTGAGCTACGTCATGGTGGGATACTCGCAGACCGGGCAGATGCGT
  VEG MIF   (101) AGCCTCTGAGCTACGTCATGGTGGGATACTCGCAGACCGGGCAGATGCGT
Consensus   (101) AGCCTCTGAGCTACGTCATGGTGGGATACTCGCAGACCGGGCAGATGCGT

                151                                                200
  GT1 MIF   (151) TTCGGCGGGAGCAGCGACCCGTGTGCGTTCATTCGCGTTGCTTCCATTGG
 ME49 MIF   (151) TTCGGCGGGAGCAGCGACCCGTGTGCGTTCATTCGCGTTGCTTCCATTGG
 RH TgMIF   (151) TTCGGCGGGAGCAGCGACCCGTGTGCGTTCATTCGCGTTGCTTCCATTGG
  VEG MIF   (151) TTCGGCGGGAGCAGCGACCCGTGTGCGTTCATTCGCGTTGCTTCCATTGG
Consensus   (151) TTCGGCGGGAGCAGCGACCCGTGTGCGTTCATTCGCGTTGCTTCCATTGG

                201                                                250
  GT1 MIF   (201) AGGCATCACCAGTTCCACGAACTGCAAAATCGCCGCTGCTCTCTCCGCTG
 ME49 MIF   (201) AGGCATCACCAGTTCCACGAACTGCAAAATCGCCGCTGCTCTCTCCGCTG
 RH TgMIF   (201) AGGCATCACCAGTTCCACGAACTGCAAAATCGCCGCTGCTCTCTCCGCTG
  VEG MIF   (201) AGGCATCACCAGTTCCACGAACTGCAAAATCGCCGCTGCTCTCTCCGCTG
Consensus   (201) AGGCATCACCAGTTCCACGAACTGCAAAATCGCCGCTGCTCTCTCCGCTG

                251                                                300
  GT1 MIF   (251) CATGCGAACGCCACCTGGGCGTCCCCAAGAACCGCATCTACACGACATTC
 ME49 MIF   (251) CATGCGAACGCCACCTGGGCGTCCCCAAGAACCGCATCTACACGACATTC
 RH TgMIF   (251) CATGCGAACGCCACCTGGGCGTCCCCAAGAACCGCATCTACACGACATTC
  VEG MIF   (251) CATGCGAACGCCACCTGGGCGTCCCCAAGAACCGCATCTACACGACATTC
Consensus   (251) CATGCGAACGCCACCTGGGCGTCCCCAAGAACCGCATCTACACGACATTC

                301                                                350
  GT1 MIF   (301) ACAAACAAGAGCCCCTCTGAGTGGGCCATGGGCGACCGAACTTTCGGCTG
 ME49 MIF   (301) ACAAACAAGAGCCCCTCTGAGTGGGCCATGGGCGACCGAACTTTCGGCTG
 RH TgMIF   (301) ACAAACAAGAGCCCCTCTGAGTGGGCCATGGGCGACCGAACTTTCGGCTG
  VEG MIF   (301) ACAAACAAGAGCCCCTCTGAGTGGGCCATGGGCGACCGAACTTTCGGCTG
Consensus   (301) ACAAACAAGAGCCCCTCTGAGTGGGCCATGGGCGACCGAACTTTCGGCTG

                351
  GT1 MIF   (351) A
 ME49 MIF   (351) A
 RH TgMIF   (351) A
  VEG MIF   (351) A
Consensus   (351) A
```

Figure 2.1a: Nucleotide alignment between MIFs of RH, GT1, ME49 and VEG.

Identical nucleotides are shown in red and conserved nucleotides are shown in aqua. Alignment shows 99.7% nucleotide sequence identity between the strains analysed. Accession Numbers (TOXO DB): GT1- TGGT1_032990; ME49- TgME49_090040; RH- GU071275; VEG- TGVEG_083780

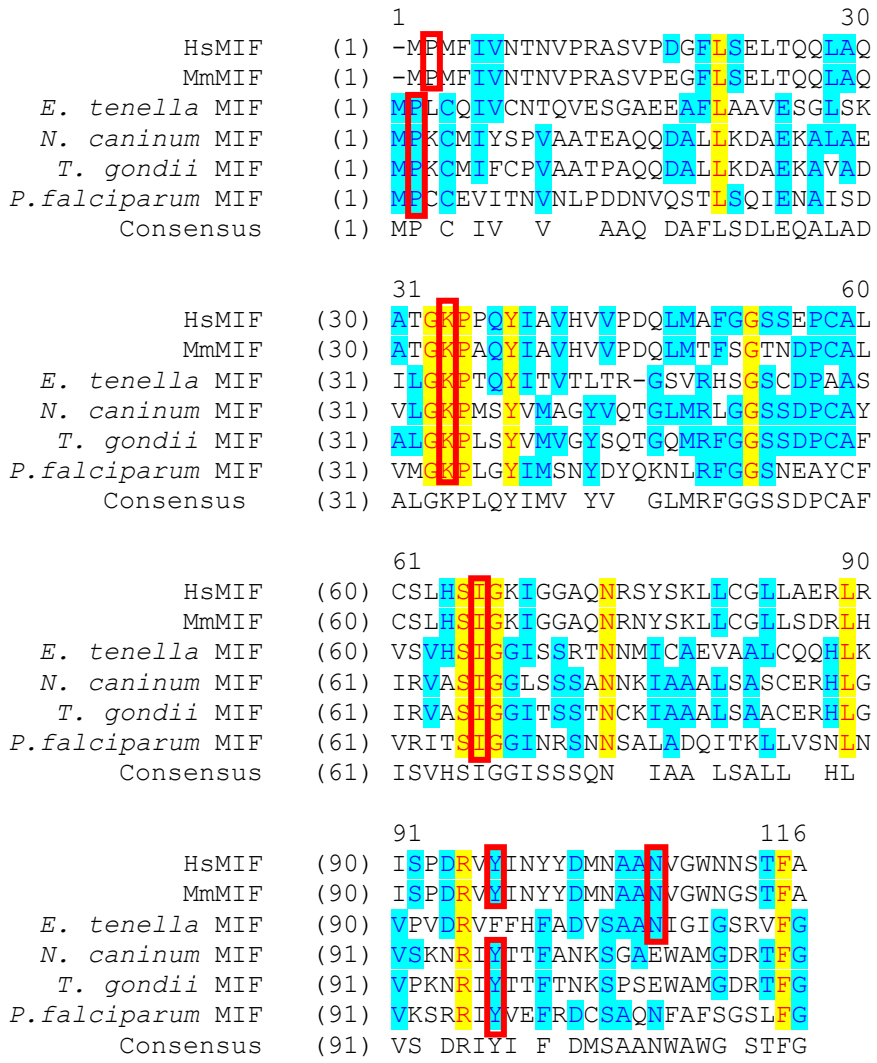


Figure 2.1b: Amino acid sequence alignment between MIFs found encoded in the genome of HsMIF, MmMIF and selected Apicomplexans.

Identical amino acids are highlighted in yellow blocks, conservative residues are highlighted in aqua blocks and non-identical amino acids are highlighted in white blocks. Accession numbers: HsMIF-NP_002406; MmMIF-NP_034928; EtMIF-ABC73371; NcMIF-NC_LIV_113040; TgMIF – ACY01255; PfMIF- PFL_1420W

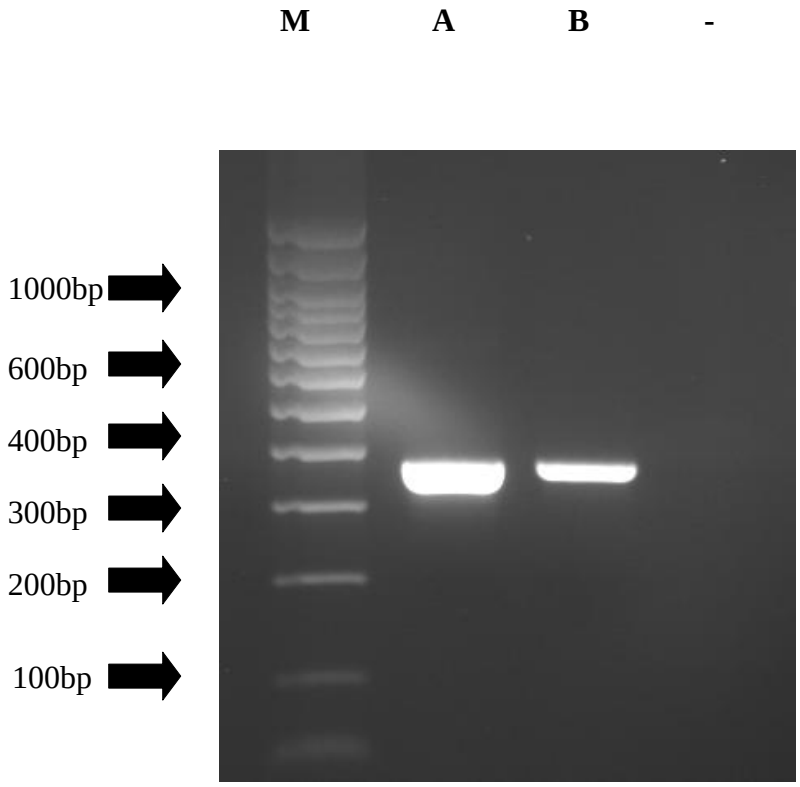


Figure 2.2: Amplification of TgMIF from tachyzoite and bradyzoite cDNA to assess expression. Amplification of TgMIF using specifically designed primers gave rise to PCR products of an expected size of 351bp. PCR reactions were performed using a standard thermal profile at an annealing temperature of 64°C. Amplification from tachyzoite cDNA (Lane A) and amplification from bradyzoite cDNA (Lane B). Negative control (-).

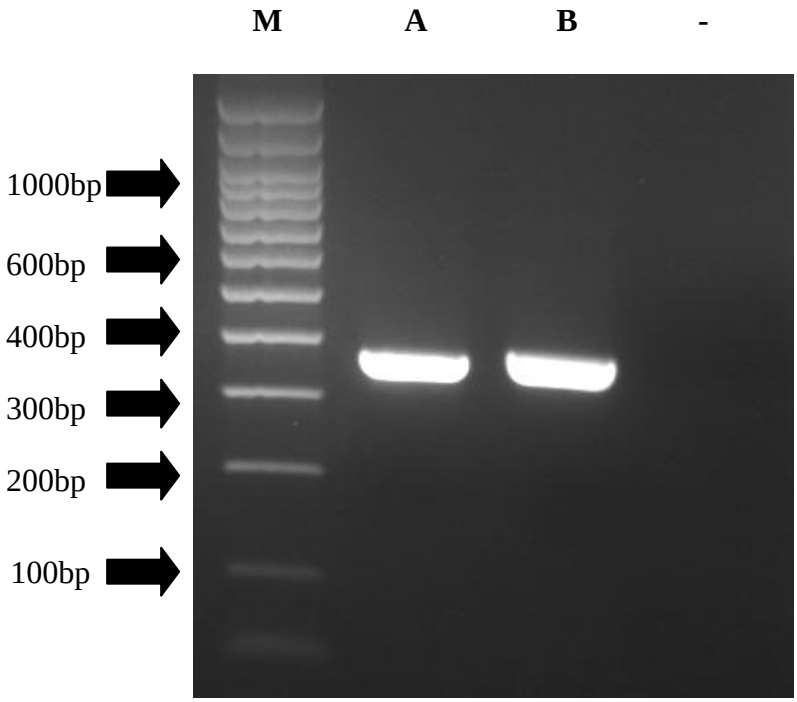


Figure 2.3a: Amplification of TgMIF from tachyzoite cDNA RH strain using high fidelity taq polymerase.

TgMIF was amplified by PCR from cDNA of tachyzoite RH strain using primers designed with restriction enzyme sites *Nde*I and *Xho*I on forward and reverse primers respectively for incorporation of PCR product into expression vector pET21a. Expected PCR product size is 351bp (Lanes A and B). Sample ran in Lane – is representative of the negative control. PCR reactions were carried out using a standard thermal profile with an annealing temperature of 64°C.

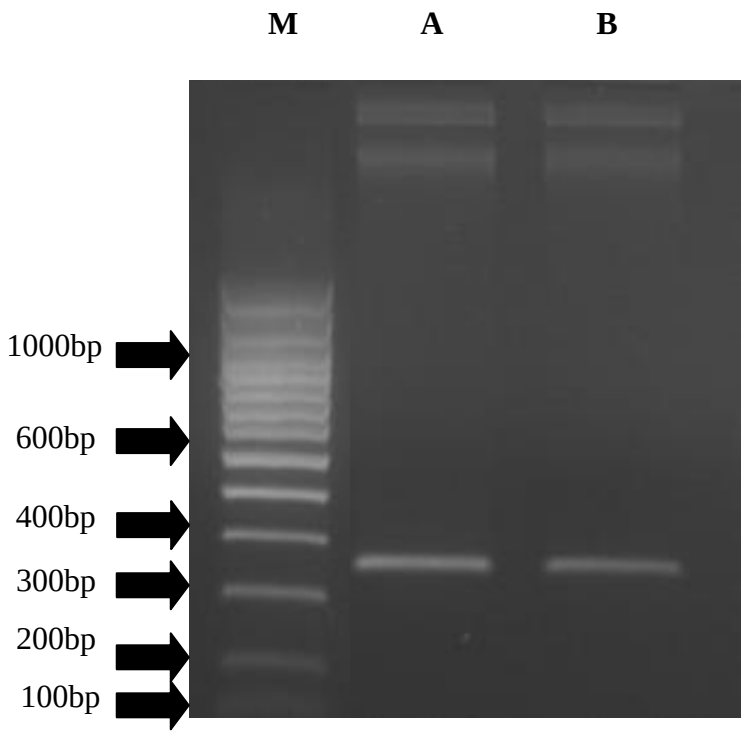


Figure 2.3b: Restriction enzyme digest of pET21a + TgMIF

Two stock plasmids (A and B) were subject to restriction enzyme digest using *Nde*I and *Xho*I to ascertain successful insertion of TgMIF (351bp) into cloning vector pET21a. Restriction enzyme digest products were ran on a 1.8% agarose gel.

size was predicted to be 351bp. Products were excised from agarose gel and ligated into the vector pET21a, which was then used to transform BL21 cells. Clones were then screened by restriction enzyme digest to ensure the presence of the TgMIF gene (Figure 2.3b). Purified plasmids were sequenced (Geneservice, Cambridge, UK) to ensure there were no amplification errors, which may affect TgMIF sequence and structure. The TgMIF sequence was deposited in GenBank (Accession number GU071275).

2.3.4 Determination of solubility of TgMIF

Protein expression was induced in BL21 cells with 1mM IPTG at 30°C overnight. Harvested cells were then sonicated twice to disrupt cell membranes and samples of soluble and insoluble fractions were collected at each step. Analysis of fractions by SDS-PAGE shows a protein of ~12.5kDa, predicted to be TgMIF, to be soluble (Figure 2.4).

2.3.5 Purification of TgMIF

Large scale expression was carried out in 1L batches following the normal expression protocol. Harvested cells were subjected to sonication and treatment with lysozyme prior to purification. Protein was purified using Ni^{2+} NTA columns with samples of flow through from the addition of cell lysates and wash steps also being collected for analysis by SDS PAGE (Figure 2.5). Flow through fraction 1, following the addition of cell lysates, contained many *E.coli* proteins which passed straight through the column as expected. A band at 12.5KDa is prominent in this lane, although, this can be accounted for due to column overloading. Analysis of flow through fraction 2, containing any unspecifically bound proteins or weakly bound his tag protein again showed a prominent band of 12.5kDa which once again can be accounted for due to column saturation.

Eluted protein was collected in 100µl fractions and purity was assessed visually via SDS PAGE analysis (Figure 2.6). A single band is visible in all samples of eluted protein at 12.5KDa.

Purified protein was probed via western blot with an anti his antibody for verification of a 12.5kDa protein containing a His tag (Figure 2.7). Again, single bands were obtained, representative of C-terminally His tagged TgMIF.

Further confirmation of the representation of the 12.5kDa band of TgMIF was sought by MALDI-TOF mass spectrometry. Mass spectrometry identified eight peptides which produced a significantly high score that assures the protein eluted in the purification procedure is TgMIF (Table 2.1).

2.3.6 Determination of TgMIF secretion

ESAg and a sample of supernatant from RH infected HFFs were probed with mouse anti-TgMIF polyclonal antibody by western blot. A single band present in lane B loaded with supernatant, indicated that TgMIF is a secreted protein (Figure 2.8).

Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Expect	Rank	Peptide
494.1669	986.3193	986.5073	-0.1880	0	53	0.0079	1	R.IYTTFTNK
566.6740	1131.3334	1131.5706	-0.2372	0	75	4.5×10^{-5}	1	K.IAAALSAACER.H
567.8000	1133.5851	1134.4764	-0.8909	0	23	7.5	1	K.SPSEWAMGDR.T
576.0835	1150.1525	1150.4713	-0.3188	0	46	0.035	1	K.SPSEWAMGDR.T + Oxidation
657.1563	1312.2980	1312.5870	-0.2890	0	98	1.6×10^{-7}	1	R.FGGSSDPCAFIR.V
697.7534	1393.4923	1393.6871	-0.1948	0	90	1×10^{-6}	1	R.VASIGGITSSTNCK.I
853.2469	2556.7189	2557.2668	-0.5478	0	74	2.1×10^{-5}	1	K.AVADALGKPLSYVMVGYSQTGMQR.F + Oxidation (M)
858.5563	2572.6469	2573.2617	-0.6148	0	52	0.0034	1	K.AVADALGKPLSYVMVGYSQTGMQR.F + 2 Oxidation (M)

Table 2.1: Mass spectrometry data

A 12.5kDa band proposed to be representative of TgMIF was excised from an SDS gel, which was then subject to MALDI-TOF mass spectrometry. Mass spectrometry analysis identified eight peptides from the sample which correlated with an entry in GenBank of a MIF homologue found in *Toxoplasma* GT1 strain.

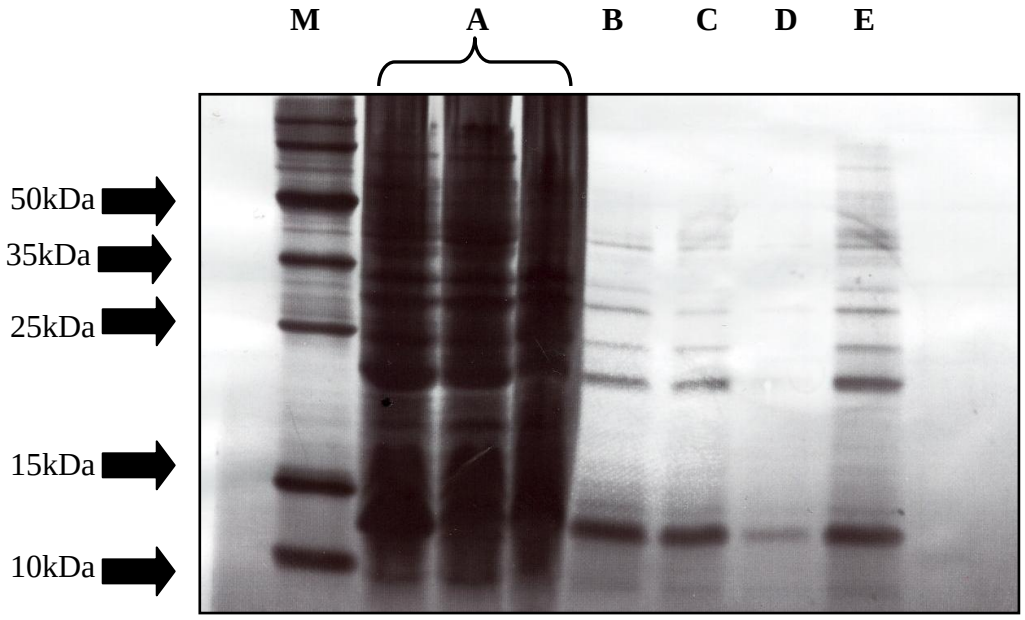


Figure 2.4: Determination of solubility of TgMIF

Expression was induced with 1mM IPTG overnight at 30°C. Cells were harvested and then subject to two rounds of sonication. Samples are shown on a 14% SDS-PAGE resolving gel which was coomassie stained. Samples from induced broth pre-sonication are shown in lane A. Insoluble and soluble fractions following the first round of sonication are shown in lanes B and C. Insoluble and soluble fractions following second round of sonication are shown in lanes D and E. Bands of 12.5KDa in lanes B→E correspond to the predicted molecular weight of TgMIF.

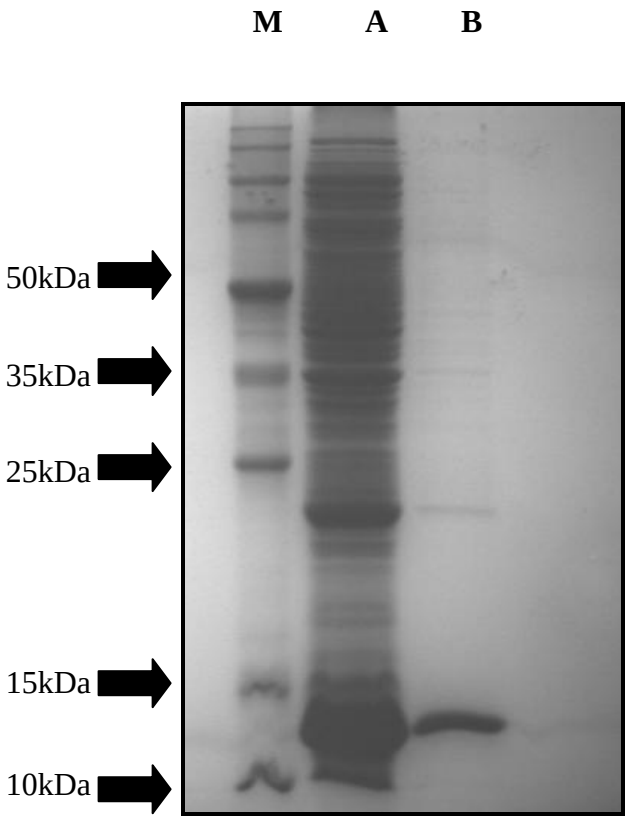


Figure 2.5:Assessment of flow through fractions from purification procedure

Protein was purified using Ni^{2+} NTA columns. Samples of flow through fractions were collected for analysis by SDS PAGE analysis. Samples are shown on a 14% SDS-PAGE resolving gel which was coomassie stained. Proteins present in the first flow through after the addition of lysate to the column are shown in lane A. Proteins in the flow through following the wash step are shown in lane B.

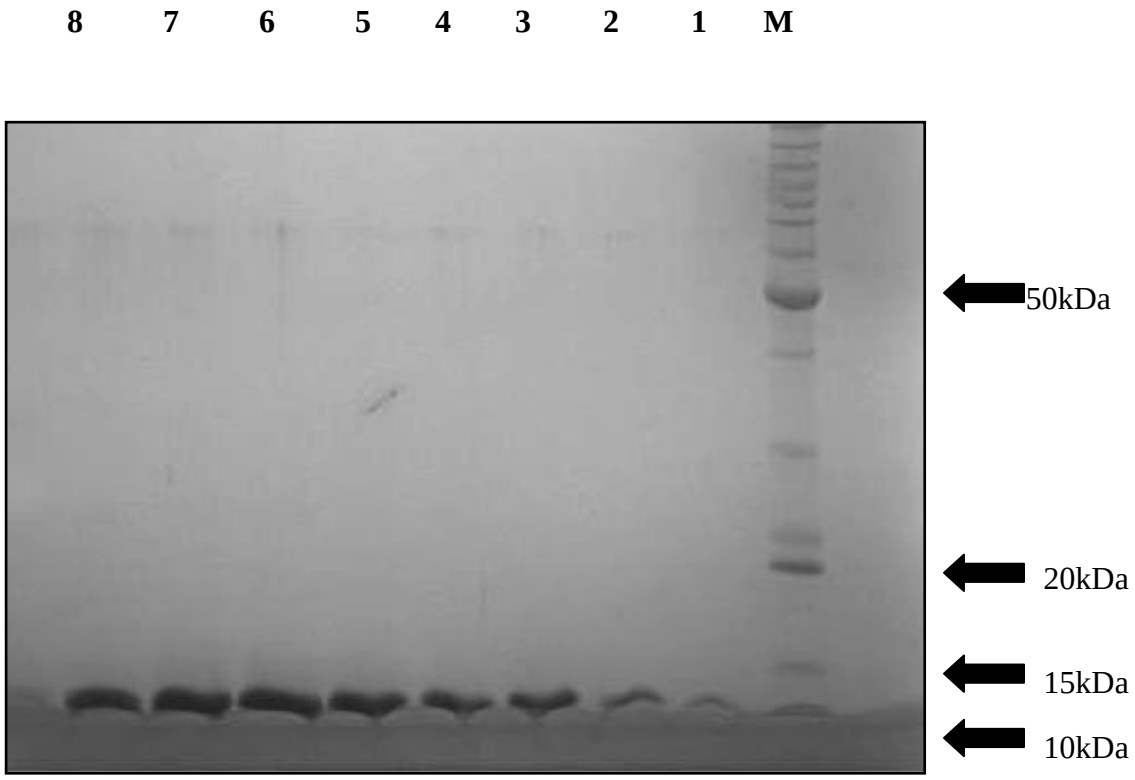


Figure 2.6: Analysis of eluted fractions of purified TgMIF

Samples of eluted protein ran on a 14% SDS PAGE resolving gel which was coomassie stained (Lanes 1→8). Bands ~12.5KDa correspond to the predicted molecular weight of TgMIF. Protein was eluted using elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM imidazole, pH8.0).

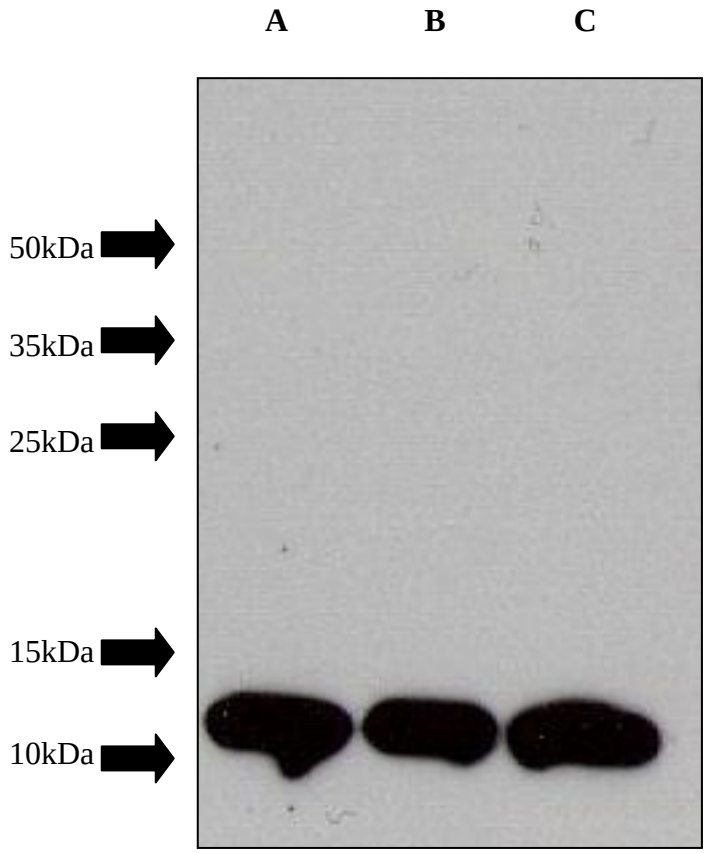


Figure 2.7: Confirmation of presence of His tag TgMIF by western blot

Samples of eluted purified protein were probed with an anti his antibody by western blot (Lanes A→C). Samples in lanes A, B and C are elutes 5,6 and 7 from the previous figure (Figure 2.6). Bands of 12.5KDa, correspond with the predicted molecular weight of TgMIF monomer.

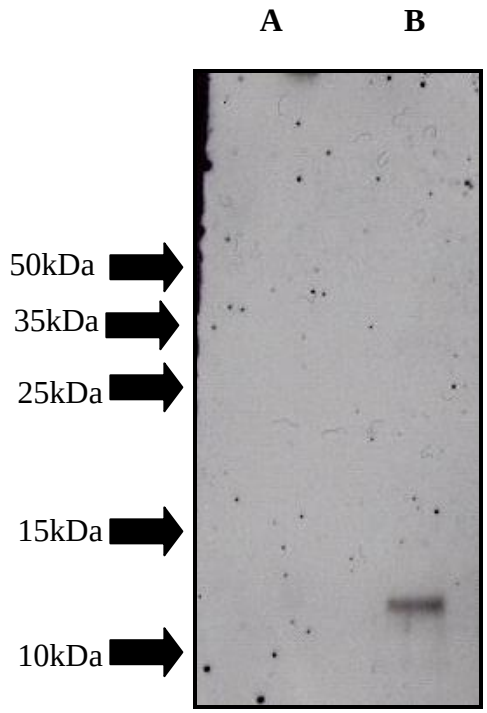


Figure 2.8 :Western blot for TgMIF secretion

Samples of ESAg(A) and supernatant from HFF's infected with *T. gondii* (RH) (B) were probed with polyclonal anti-TgMIF antibody. The presence of a 12.5KDa protein in lane B, indicates that TgMIF is a secreted protein.

2.4 Discussion

Since its discovery in the late 1960's, studies into the function of MIF have detailed a role for this ubiquitous pro-inflammatory cytokine in numerous human infections and diseases, such as rheumatoid arthritis, septic shock and cancers (Reviewed Bach *et al*., 2008). However, since the late 90's to the present day several studies have identified the presence of MIF homologues in many parasitic species including helminths and protozoa, many of which have been now characterised (Tan *et al.*,2001, Augustijn *et al.*,2007, Miska *et al.*, 2007, Kamir *et al.*, 2008)

Analysis of the *T. gondii* genome reveals one homologue of human MIF, whereas in other protozoan species e.g. *L. major*, two MIF orthologs are encoded in the genome (Kamir *et al*., 2008). Bioinformatical analysis of four different *T. gondii* strains from three major lineages: GT1, VEG, ME49 and RH (as identified by this project) shows great conservation of MIF within these strains. In fact, 99.7% sequence identity is associated between these strains, with a single substitution (thymine for cytosine) at position 21 being the only accountable difference. This substitution, however, does not result in an amino acid change and a phenylalanine, at this position, is present in all *T. gondii* strains sequenced so far. Therefore, at both the nucleotide and protein level, the MIF homologue found in *T. gondii* is conserved.

As well as *T. gondii*, MIFs have also been described in several other Apicomplexa including *P. falciparum, E. tenella* and *N. caninum* (Augustijn *et al.*, 2007, Miska *et al.*, 2007). Alignments of the TgMIF amino acid sequence with Human MIF (HsMIF), Mouse MIF (MmMIF) and the aforementioned Apicomplexa reveals 25.9%, 23.3%, 43%, 32.6% and 76.6% sequence identity, respectively. In addition, these alignments highlight 15 invariant residues. Of note, the greatest sequence identity is observed between *T. gondii* and *N. caninum*. However, this is not unexpected as several papers have reviewed the similarities between these two species with some even debating the possibility that *Neospora* could potentially be a strain of *Toxoplasma* (Reviewed Mehlhorn and Heydorn, 2000, Siverajah *et al.*, 2003). Finally, it should be noted that MIF homologues are not found in all Apicomplexa and are absent from *Cryptosporidium parvum* and *Theileria annulata* (Augustijn *et al*., 2007). Assuming selective pressure resulted in the deletion of this gene from the genomes of the aforementioned species, this would suggest that at some point the gene product became redundant in these parasites.

Despite somewhat lower identities recorded between *T. gondii* and humans, it is notable that several of the most important residues associated with MIF function have been evolutionarily

conserved in *T. gondii*. Of interest, the first proline associated with tautomerase activity, is found conserved in all homologues to date (Figure 2.1b), highlighting an indication of its potential evolutionary importance. Significantly, despite only moderate homology observed between homologues when compared to HsMIF, greatest conservation is found around active site residues associated with tautomerase activity i.e. positions 1, 33, 65 and 95, suggesting perhaps a shared function between these homologues.

Furthermore, a CXXC motif found in both human and mouse MIF at positions 58→61 which mediates MIF oxidoreductase activity (Kleemann *et al.,* 1998), is partially conserved in several homologues including *T. gondii* and *Eimeria*. Partial conservation of this motif has been shown to be detrimental to optimal oxidoreductase activity in other homologues, including *Plasmodium* and *Leishmania* (Augustijn *et al.,* 2007, Richardson *et al.,* 2009). This aspect of MIF function in *T. gondii* will be discussed in detail at a later point.

Low sequence identity can sometimes be an indication of poor structural conservation. Crystal structure studies have been carried out on various MIFs and to date the structures have been solved for human, *L. major, B. malayi* and *Ancylostoma ceylanicum* (Sun *et al.,* 1996, Kamir *et al.,* 2008, Zang *et al.,* 2002, Cho *et al.,* 2007). In many of these examples, a low sequence identity with HsMIF was observed, although, structural conservation with its mammalian counterpart was maintained (Augustijn *et al.,* 2007, Kamir *et al.,* 2008, Zang *et al.,* 2002). Therefore, it is also possible that structural conservation with HsMIF will be evident in the *T. gondii* homologue of MIF despite sharing only 25.9% sequence identity.

TgMIF is expressed in both tachyzoite and bradyzoite stages of the *T. gondii* life cycle at the mRNA level. Further studies would need to be carried out in order to verify that TgMIF is also expressed at the protein level and indeed also to observe any potential differences in stage specific expression. However, MIF expression does appear to vary in different stages of the life cycle in other parasitic homologues. Kamir *et al*., (2008) found differences in *L.major* MIF (LmMIF) levels in mRNA transcripts from procyclics, metacyclics and amastigotes with a notable increase being observed in procyclics. This would indicate the importance of LmMIF in survival within the sand fly as opposed to the human host. In addition, Augustijn *et al*., (2007) showed the *P.berghei* MIF (PbMIF) is present in all parasite stages of the *Plasmodium* life cycle although, a peak is observed at the trophozoite stage. Furthermore, PbMIF was found secreted in the cytoplasm of both the parasite and infected erythrocyte.

Expression of MIF has been shown to vary in other parasite species including *E. tenella* (Miska *et al.,* 2007). *E. tenella*, a closely related parasite to *T. gondii*, encodes one copy of MIF in its genome and is expressed at the mRNA level in greatest quantities in merozoites whereas low or virtually undetectable levels were recorded in oocysts and sporozoites. Of interest, immunofluorescence staining indicated that although EtMIF is found distributed throughout the cytosol, it is primarily concentrated to the apical end of the parasite (Miska *et al.,* 2007).

Bioinformatical analysis reveals that TgMIF, like mammalian MIF, does not have a classical von Heijne secretory peptide. Furthermore, TgMIF does not possess any targeting sequences for either the micronemes or the rhoptries. Therefore, *Toxoplasma* cell biology would dictate that this protein should be secreted via the dense granules. In order to determine the mechanism by which TgMIF is secreted, recombinant TgMIF was used to generate polyclonal antibodies to TgMIF. Antibodies were then used in a western blot to detect TgMIF secretion in the ESAg or supernatant from an infected flask of HFFs. Specifically, the absence of a band representative of TgMIF in the ESAg sample indicates that TgMIF is not secreted via the dense granules. However, the presence of a band in the supernatant sample from a flask of HFFs infected with tachyzoites, confirms that TgMIF is secreted. This therefore suggests that TgMIF secretion is similar to that of mammalian MIF in that secretion is via a non-classical pathway. Non classical protein secretion is not unique to MIF and to date there are approximately 20 different proteins secreted in a non classical manner including IL-1β, Thioredoxin (Trx) and fibroblast growth factor 2 (FGF2) (Rubartelli *et al.*, 1990, Rubartelli *et al.*, 1992, Renko *et al.*, 1990). Inhibition studies have demonstrated that MIF secretion was significantly reduced following treatment with glyburide, an ABC transporter inhibitor (Flieger *et al.*, 2003). More specifically, further investigations detailed a role for the golgi-associated protein p115 in mediating human MIF secretion (Merk *et al.*, 2009). p115 is a highly conserved protein amongst mammals, however, alignments of p115 homologues from a variety of different species, shows this protein to be highly divergent with sequence identity ranging from 10.8% in *Giardia lamblia* and *Saccharomyces cerevisiae* to 32.4% in *Schistosoma mansoni* in comparison with human p115 and murine p115 (see Appendix C). Consequently, a BLAST search of ToxoDB using these sequences produced no significant hits. Furthermore, a search for p115 homologues in other protozoan genomes e.g. *Plasmodium, Leishmania, Eimeria* species, did not produce any significant hits. A BLAST search using the p115 sequence of the highly divergent *Mycoplasma* produced significant hits in the *Toxoplasma*

genome with a putative protein involved in the structural maintenance of chromosomes. Therefore, at this point, the presence of a p115 homologue in the *Toxoplasma* genome cannot be confirmed. Furthermore, since p115 cannot be found in the genomes of several other protozoa, it is possible that these MIF homologues may be secreted independently from p115.

Chapter 3

Characterisation of TgMIF enzymatic activities

3.0 Abstract

The tautomerase and oxidoreductase catalytic activities of MIF are unique amongst cytokines. Identification of an N-terminal proline-1 dependent tautomerase activity and CXXC dependent oxidoreductase activity associated with MIF, led to speculation that these enzyme activities were responsible for the biological activities of MIF. Analysis of the TgMIF sequence reveals conservation of the N-terminal proline and partial conservation of the CXXC motif. In the present study, TgMIF tautomerase activity was shown to be significantly lower than that of its human counterpart, HsMIF. In addition, oxidoreductase activity was shown to be absent from TgMIF.

3.1. Introduction

A unique feature of MIF, unlike any other cytokine, is the presence of two catalytic activities: tautomerase and oxidoreductase activities. MIF tautomerase activity was first described by Rosengren *et al.,* (1996), who isolated a tautomerising agent from bovine eye lens, which was subsequently identified as MIF. Since then, many MIF proteins that exhibit tautomerase activities have been identified from many tissues and from a variety of different species (Sun *et al.,* 1996, Suzuki *et al.,* 1996, Han *et al.,* 2004, Du *et al.,* 2006).

MIF tautomerase activity is associated with the N-terminal proline – 1 (Bendrat *et al.,* 1997). As such, this residue is conserved throughout all species characterized to date and the mutation of which in murine MIF, results in complete loss of tautomerase activity (Bendrat *et al.,* 1997, Nishihira *et al.,* 1998). Furthermore, there is also suggestion that tautomerase activity drives MIF pro-inflammatory activities (Al-Abed *et al.,* 2005).

The oxidoreductase activity of MIF is less well characterised. However, in human MIF, it has been shown to involve the reduction of insulin in a reaction dependent on the formation and reduction of disulphide bridges via a series of oxidation and reduction reactions involving a reducing agent such as glutathione (Kleemann *et al.,* 1998). As with other thiol protein oxidoreductases, this catalytic activity has been shown to be dependent on two cysteine residues contained within the characteristic glutaredoxin/thioredoxin active site motif, CXXC, in this instance, at amino positions 58 and 61 that is essential for catalysing redox reactions (Kleemann *et al.,* 1998).

In parasitic species, tautomerase activity has been measured in many MIF homologues. However, there are few reports of a functional oxidoreductase activity in these homologues (Tan *et al.*, 2001, Cho *et al.*, 2007, Richardson *et al.*, 2009).

The aim of the present study was to assess the biochemical properties of TgMIF, determine its tautomerase and oxidoreductase activities if any, and to determine the kinetic properties of these activities. TgMIF was expressed as a C-terminal 6x His tag recombinant protein and purified as described in chapter 2.

3.2. Materials and Methods

3.2.1Tautomerase assays

Tautomerase activity of recombinant TgMIF was assessed as previously detailed by Pennock *et al.,* (1998) and a standard assay mixture contained sodium phosphate buffer (10mM, pH 5.8) containing 1mM EDTA, 48µl L-DOPA methyl ester (10mM) (Sigma-Aldrich) and 32µl sodium periodate (20mM) (Sigma-Aldrich) in a final volume of 1ml. Sodium phosphate buffers were prepared as described in the appendix and the pH was set as required by the experiment detailed below. All buffers and reagents were sterile filtered by a 0.2µm filter (Millipore, Watford, UK) prior to use. The assay components were pre-incubated for 5 min at 37 °C, before initiating the reaction by the addition of 80µg TgMIF or 0.5µg HsMIF (R&D Systems). Reaction mixtures were thoroughly mixed by pipetting. The basis of the assay involves L-DOPA which is yellow in colour and is converted to colourless DHICA. This reaction is detectable spectrophotometrically at 475 nm and the colour change during the initial rate is proportional to the concentration of L-DOPA converted. Enzyme activity was monitored for 1000 seconds at 37 °C. A similar reaction containing no enzyme was used in control experiments. Specific activities were calculated from the linear rate during the first 90 seconds of the reaction. The extinction coefficient of L-DOPA was $3700M^{-1}$ cm^{-1}. Assays were repeated at least three times.

For kinetic studies, L.DOPA was prepared in a range of concentrations from 1mM to 20mM. Assays were performed as described above in prewarmed sodium phosphate buffer containing EDTA, pH5.8 and 20mM sodium periodate. Assays were repeated at least three times.

3.2.2 Oxidoreductase assays

Oxidoreductase activity was assessed using a method previously described by Kleemann *et al.,* (1998), and a standard assay carried out in a plastic cuvette (Fisher, Loughborough, UK) containing sodium phosphate buffer (pH7.2, 10mM), 2mM EDTA 10µl of glutathione (100mM) and 50µl bovine pancreas insulin (0.5mg/ml) in a final volume of 1ml. This is a cycling reaction in which the initial rate is proportional to the concentration of glutathione. The assay components were preincubated with insulin for 5 min at 37 °C, before the reaction was initiated by the addition of 80µg TgMIF or 0.5µg HsMIF. Enzyme activity was monitored as the increase in absorbance at 650 nm due to the formation of insulin. Experimental controls

had no enzyme added to the standard assay components. Assays were repeated at least three times.

3.2.3 Statistical Analysis

Data was interpreted using GraphPad Prism Version 5.0 and GraFit 5 where all data is shown as a mean ± SE where n=3. Significant differences were determined using students t-test where ***$p<0.0001$ **$p<0.001$ *$p<0.05$ is significant.

3.3 Results

3.3.1 Determination of optimum pH of sodium phosphate buffer

Recombinant TgMIF was generated to elucidate potential enzyme activity. In order to assess TgMIF tautomerase activity, it was necessary that the optimum conditions for the assay be established. This would ensure that there were no spontaneous reactions as a result of the substrates used. Tautomerase activity was optimised first in a standard assay in the absence of protein using sodium phosphate buffers prepared across a variety of pH ranging from 5.8 to 7.0. Spontaneous tautomerase activity was evident throughout the functioning pH range of the sodium phosphate buffers used, but least at pH5.8. In addition, the recorded activity in these reactions increased between pH5.8 and 7.0, indicative of a spontaneous reaction that was dependent on pH. Therefore, pH5.8 was chosen as the optimum pH. Ideally, when conducting an enzyme kinetic study, a pH range should be assayed both above and below the selected pH. However, the pH range at which sodium phosphate buffering capacity is functional is pH5.8 – 8.0 (Dawson *et al.,* 1986). Therefore a second buffer was selected to cover the overlap at lower pH. A significant background activity was observed with citric acid phosphate buffer over a pH range from 5.6 to 6.0 under the same conditions described above whilst the omission of EDTA had no effect on the activity observed. Furthermore, L.DOPA methyl ester tautomerisation was not facilitated in these buffers (data not shown). In addition, assays were also carried out in a potassium phosphate buffer (Bendrat *et al.,* 1997, Lubetsky *et al.,* 2002), however a spontaneous reaction occurred which was so high that it was difficult to differentiate between the spontaneous reaction and the enzyme catalysed reaction (data now shown). To our knowledge, no alternative buffer for MIF tautomerisation reactions has been described and as a result assays were carried out only in sodium phosphate pH5.8 (Figure 3.1).

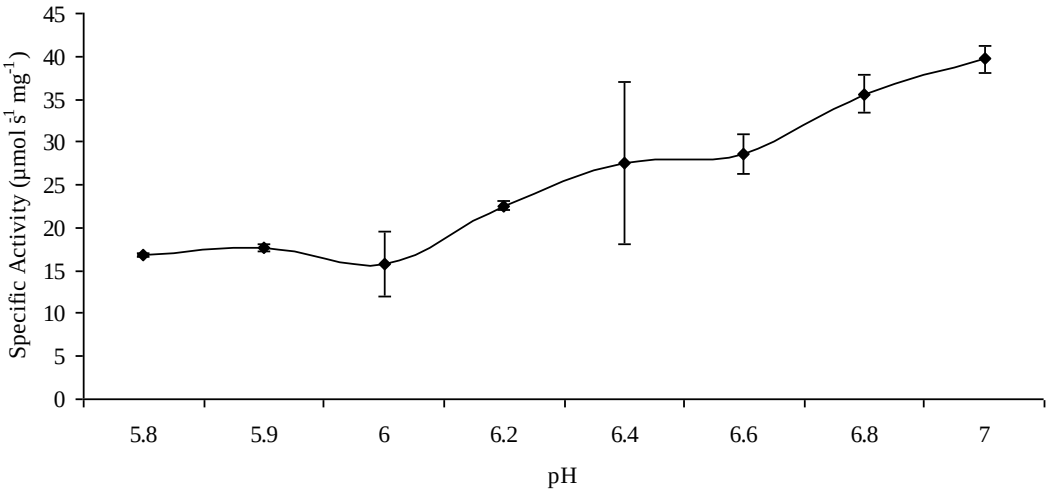


Figure 3.1 : Determination of optimum pH of sodium phosphate buffer.

pH assays were performed in the absence of protein. The optimum pH was determined to be pH5.8 to eliminate the possibility of enzyme independent spontaneous reactions, associated with higher pH. Assays were carried out at 37°C in the presence of 20mM sodium periodate, 10mM L.DOPA and 10mM sodium phosphate buffer. Assays were repeated at least three times.

3.3.2 Determination of optimum storage conditions

Studies were carried out to determine the optimum storage conditions and enzymatic lifespan of MIF protein. Aliquots of TgMIF protein at ~6mg/ml were stored at 4°C, -20°C and -80°C in both the presence and absence of 50% glycerol. Aliquots of recombinant TgMIF from each storage condition were assayed for tautomerase activity daily for 14 days. Recombinant TgMIF stored at 4°C quickly lost ~35% of tautomerase activity within 24 hours post elution both with and without glycerol but retained ~60% of its original activity for 11 days after which its activity diminished (Figure 3.2A). At -20°C, glycerol added to TgMIF at 50%, had a stabilizing effect on the tautomerase catalytic activity of this protein for ~2-3 days and thereafter lost an additional 35% of its tautomerase activity over the period of observation (Figure 3.2B). However, tautomerase activity in the absence of glycerol decreased steadily by 40% during the same period. Finally, protein stored at -80°C remained stable with over 60% activity for the longest period of time, ~4days post elution with close to 100% activity being retained for the first 3 days (with or without glycerol (Figure 3.2C). In addition, tautomerase activity following storage in both the presence and absence of glycerol resulted in a 40% reduction in activity by day 7. However, no further decrease in activity was observed for the glycerol treated sample for the rest of the duration of the study whereas activity in the absence of glycerol decreased after day 7 (Figure 3.2C). There were no obvious preservative benefits observed by the addition of 50% glycerol in the short term. Therefore, with this is mind, TgMIF used for biochemical assays was stored at -80°C without glycerol for no more than 4 days post elution.

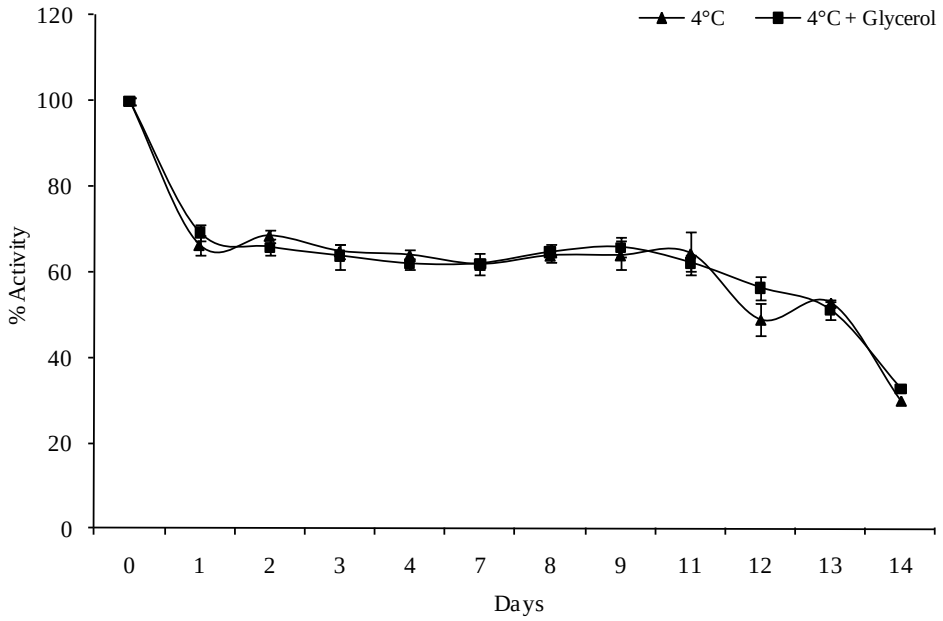
3.3.3 TgMIF tautomerase kinetics

Purified recombinant TgMIF was enzymatically active towards L.DOPA methyl ester with a specific activity of 1476 μmol/min/mg of protein, a value that falls within the range reported previously for *L. major* MIFs (Figure 3.3C) but 81 and 91 fold lower than that reported for murine and human MIFs (Richardson *et al.,* 2009) (Figure 3.3A). TgMIF kinetics parameters were measured by recording tautomerase activity in the presence of a variety of concentrations of L.DOPA methyl ester ranging from 1 to 20mM (Figure 3.3B). TgMIF has a specific activity of 1476 μmol/min/mg and K_m and V_{max} values were determined to be 9.6mM and 1800μmol/min/mg respectively (Figure 3.3B; Table 3.1). The resultant apparent Kcats and Kcat/Km were 0.135 S^{-1} and 13.99 S^{-1} M^{-1} respectively for L.DOPA.

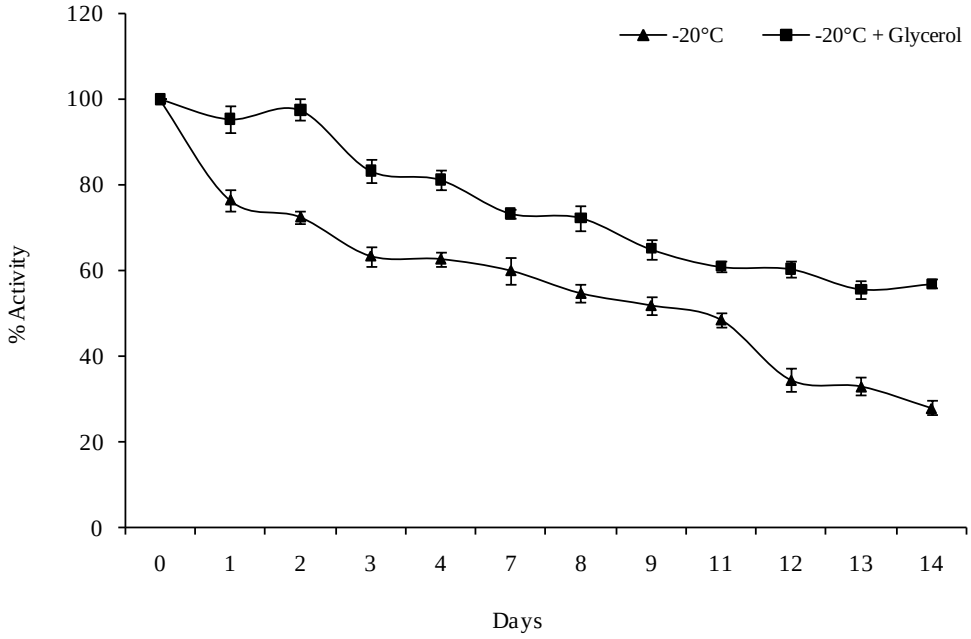
3.3.4 Measurement of TgMIF oxidoreductase activity

Human MIF has been shown to exhibit thiol-protein oxidoreductase activity in the insulin reduction assay (Kleemann *et al.,* 1998). TgMIF was therefore assayed for oxidoreductase activity. Initial assays revealed an apparent TgMIF oxidoreductase activity. However, similar rates of reactions were observed in experimental controls and consequently the apparent TgMIF oxidoreductase activity was actually due to an enzyme independent activity as a result of an excessive L-glutathione concentration which diminishes the requirement for TgMIF in the reaction cascade (Figure 3.4A). Optimisation assays led to the selection of 1mM L-glutathione for further oxidoreductase assays (Figure 3.4B). TgMIF and HsMIF oxidoreductase activity was recorded over a period of 110 minutes. 0.9µM Trx was used as a positive control (Kleemann *et al.,* 1998). TgMIF oxidoreductase activity was not detectable and comparable to the no enzyme controls. HsMIF activity was evident but reaction onset was delayed in comparison to Trx (Figure 3.4C).

A


4°C
4°C + Glycerol
120
100
80
60
40
20
0
% Activity
0
1
2
3
4
7
8
9
11
12
13
14
Days

B


-20°C
-20°C + Glycerol
120
100
80
60
40
20
0
% Activity
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1
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13
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Days

C

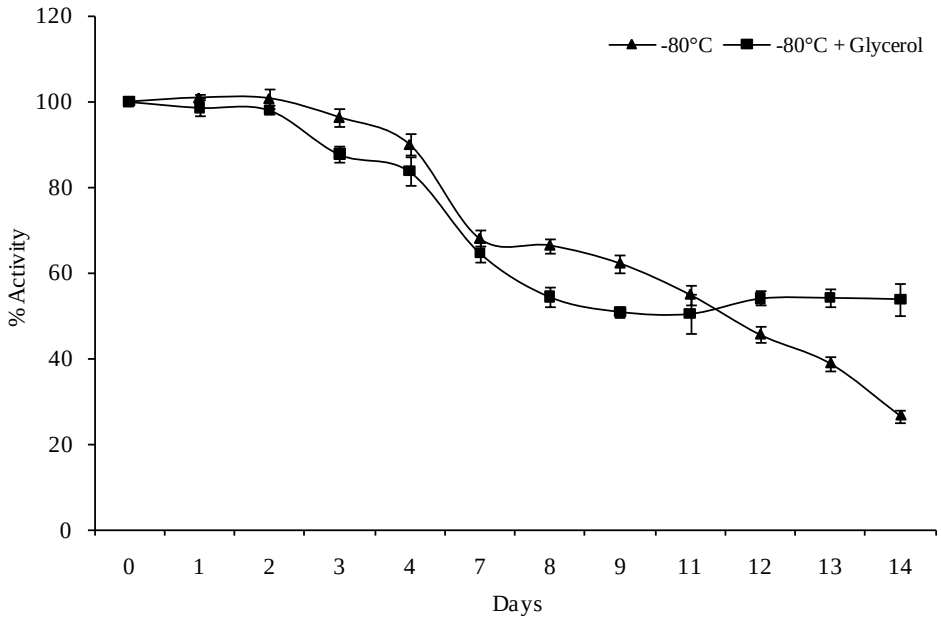
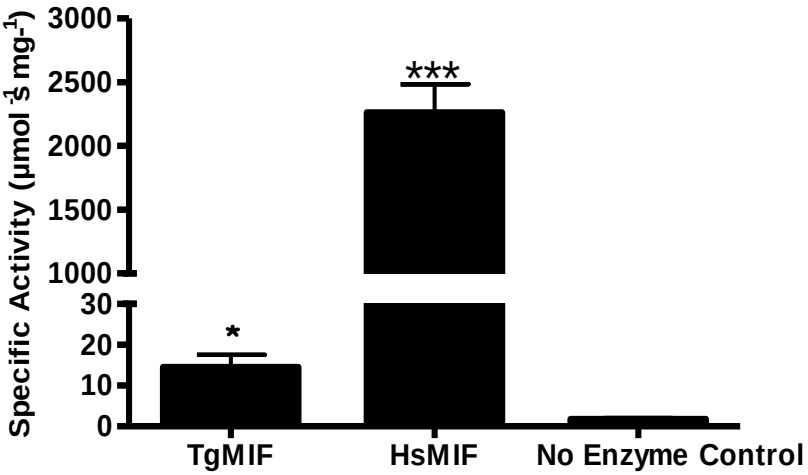


Figure 3.2: Determination of the optimum storage conditions

Protein aliquots were stored at ~6mg/ml at 4°C (Figure 3.2A), -20°C (Figure 3.2B) and -80°C (Figure 3.2C) in both the presence and absence of glycerol. Protein was then assayed for tautomerase activity daily for 14 days. Assays were repeated at least three times.

A



B

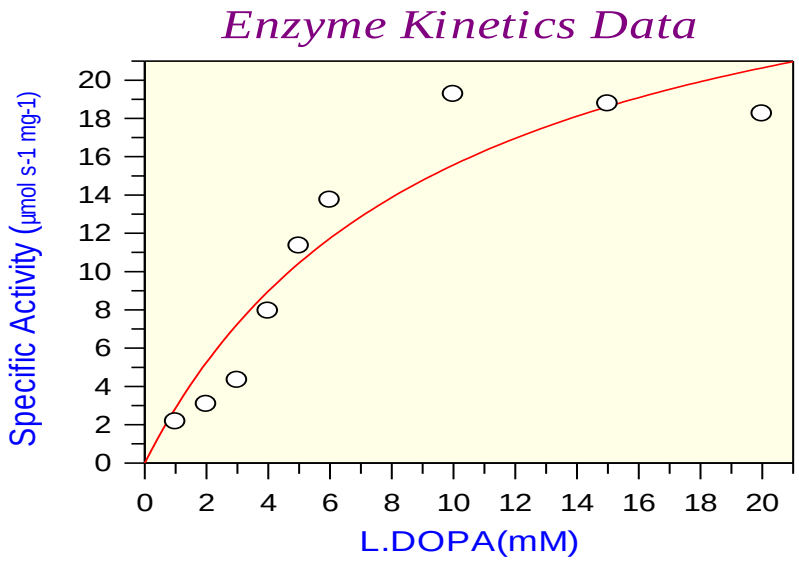


Figure 3.3: Measurement of TgMIF tautomerase kinetics.

TgMIF has tautomerase activity (Figure 3.3A) and subsequently TgMIF tautomerase kinetics were measured towards L.DOPA methyl ester (Figure 3.3B). Assays were repeated at least three times. *p<0.05 ***p<0.0001

	Specific Activity (μmoles/min/mg)	**K_m** (μM)	**V_{max}** (μmoles/min/mg)	**K_{cat}** (S^{-1})	**K_{cat}/K_m** ($S^{-1}M^{-1}$)
TgMIF ∞	1476	9600	1800	0.135	13.99
TsMIF *	3500	5600	4000	8100	1.6×10^6
HsMIF *	588	5000	650	1340	0.27×10^6
HsMIF §	3350	-	-	-	-
HsMIF ∞	135,720	-	-	-	-
MmMIF †	120,600	-	-	-	-
LmMIF †	4320	-	-	-	-

Table 3.1 Kinetics of L.DOPA methyl ester tautomerase activity of MIF homologues

Tautomerase activity for L.DOPA methyl ester of TgMIF is reduced compared to specific activities of other parasite homologues and human MIF.[1]

[1]*Tan *et al.,* 2001, § Kamir *et al.,* 2007,∞ This study, †Richardson *et al.,* 2009

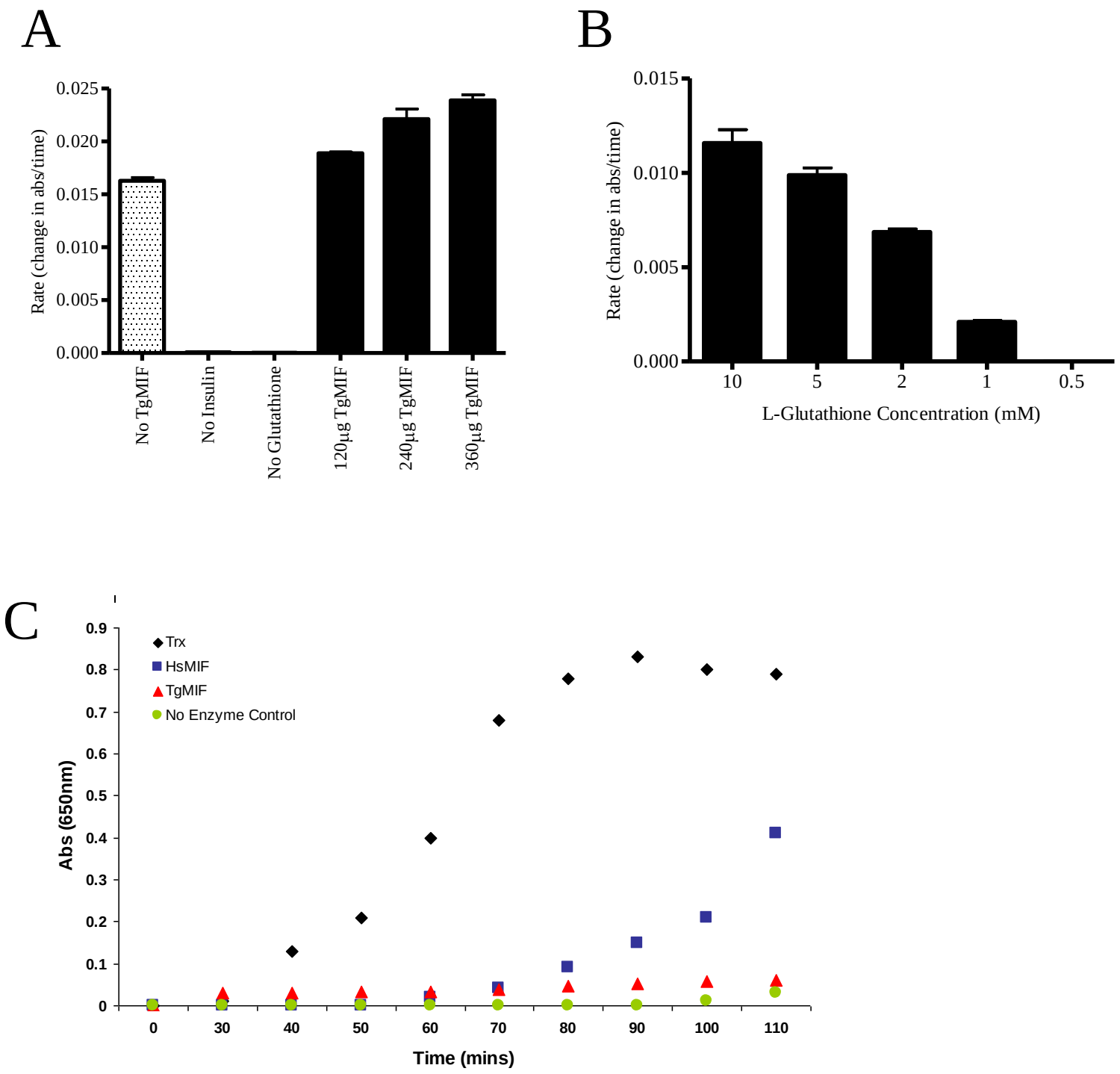


Figure 3.4 Determination of TgMIF oxidoreductase activity

Initial assays showed oxidoreductase activity was not an enzyme dependent reaction (Figure 3.4A) and therefore further optimisation of substrate concentration was carried out (Figure 3.4B). TgMIF oxidoreductase activity was then measured using the optimised assay (Figure 3.4C). Assays were repeated at least three times.

3.4. Discussion

The tautomerase and oxidoreductase activities of mammalian MIF have been well characterised with many studies indicating biological roles for these enzymatic functions (Dios *et al.,* 2002, Lubetsky *et al.,* 2002, Al-Abed *et al.,* 2005). Consequently, description of the enzyme characteristics of the newly identified TgMIF homologue, are described herein.

C-terminal His tagged recombinant TgMIF was used in enzyme assays as previous studies have indicated that N-terminally modified protein severely impedes MIF tautomerase activity (Richardson *et al.,* 2009). Furthermore, in this study, tautomerase activity was measured by the conversion of L.DOPA methyl ester to DHICA. Though other substrates have been used to characterize MIF tautomerase activity e.g. *p*-hydroxyphenylpyruvate (Tan *et al.,* 2001, Zang *et al.,* 2002), the specific activities measured have been very low, thus suggesting they are less well utilised by MIF. As such, L-DOPA methyl ester was adopted in this study, but neither L. DOPA methyl ester nor *p*-hydroxyphenylpyruvate is believed to be the natural substrate for MIF tautomerase activity *in vivo*. However, until the true natural substrate for this activity is identified, the relevance of these results in a biological context is unknown, however, a similar mechanism is likely with its *in vivo* natural substrate. Typically, parasite MIFs have a reduced ability to tautomerise non-physiological substrates compared with mammalian MIF (Zang *et al.,* 2002, Richardson *et al.,* 2009, Augustijn *et al.,* 2007, Cho *et al.,* 2007). However, it is possible that parasite homologues of MIF, such as TgMIF, may tautomerise the natural substrate equally well as mammalian MIFs.

The tautomerase activity of HsMIF has been characterised previously (Rosengren *et al.,* 1996). However, extensive variability in the measured specific activities of human MIF towards L.DOPA methyl ester have been recorded (Table 3.1). Indeed the specific activity of human MIF has been reported to vary between 588 (Tan *et al.,* 2001) to 135,720µmols/min/mg (this study). Closer inspection of assay protocols for the aforementioned studies suggests that differences in buffers, pH, temperature and instrumentation could account for variation in the results obtained. (Tan *et al.,* 2001, Kamir *et al.,* 2008). Therefore, subsequent reference to HsMIF in this chapter will be made with regard to the activities recorded as a result of the present work, (135,720 µmols/min/mg), as the exact same parameters for defining this activity were also used for TgMIF characterisation.

TgMIF has a measured specific activity of 1476µmol/min/mg towards L.DOPA methyl ester, which was approximately 81 and 91 fold less than that of MmMIF and HsMIF respectively (Richardson *et al.,* 2009) but comparable to LmMIF tautomerase activity, also 27 fold and 31 fold lower than the MmMIF and HSMIF respectively (Richardson *et al.*, 2009).

Characterisation of TgMIF tautomerase kinetic parameters reveals that TgMIF and HsMIF had comparable Kms, being 9.6mM and 5mM respectively (Tan *et al.,* 2001). Consequently, HsMIF has a greater affinity for L.DOPA methyl ester than TgMIF. Furthermore, comparison between the Kcat/Km of TgMIF and HsMIF highlights HsMIF as a much more efficient enzyme which has greater turnover of substrate per molecule of enzyme. Unfortunately, there is a significant lack in fully comprehensive enzyme kinetic studies in other MIF homologues, with the most detailed study to date being performed with *T.spiralis* MIF (TsMIF) (Tan *et al.*, 2001). Comparison of TgMIIF with TsMIF indicates TsMIF has a much greater specific activity (3500µmol/min/mg) than TgMIF. In fact, this activity in TsMIF is 6 fold greater than HsMIF (Tan *et al.,* 2001). Furthermore, TsMIF has greater affinity for L.DOPA methyl ester and subsequently a higher turnover of substrate thus making TsMIF a much more efficient enzyme than TgMIF.

Initial assays to characterise TgMIF oxidoredcutase activity showed this catalytic activity to be non-TgMIF dependent. As expected, the reaction was shown to be dependent on the addition of insulin and glutathione. Furthermore, the addition of 2-3 times more enzyme had no significant effect on the rate of reaction measured. Measurement of a weak activity in both the presence and absence of enzyme prompts suggestion of a poor catalytic activity for TgMIF oxidoreductase activity, even up to two hours post addition of enzyme in an optimised assay.

It was hypothesised that the measured activity in the no enzyme control was due to the high glutathione concentration in the reaction (4.5mM) as detailed by Kleemann *et al.,* (1998). Subsequently a series of optimisation assays led to the selection of 1mM glutathione for further oxidoreductase assays. In comparison with measured activity of a natural thiol protein oxidoreductase such as Trx, HsMIF has a measurable oxidoreductase activity although the onset of this reaction is delayed. However, TgMIF displays virtually no catalytic activity at all up to 2 hours post addition of enzyme. This lack of activity was not unexpected since previous reports have indicated that the presence of two cysteines at positions 57 and 60 are essential for mediating this activity, with greatest importance being placed on the second cysteine

(Kleemann *et al.*, 1998). Therefore, the absence of the second cysteine in TgMIF suggested that oxidoreductase activity in this homologue would be weakened if not non-existent. This is in keeping with observations made in other homologues thus far studied (Augustijn *et al.*, 2007, Richardson *et al.*, 2009). For example, PfMIF which has one conserved cysteine, exhibits an oxidoreductase activity which is 20% of the recorded activity for HsMIF (Augustijn *et al.*, 2007). However, even HsMIF does not have a strong oxidoreductase activity, which may indeed raise the possibility that this activity may be redundant in comparison to tautomerase activity.

Chapter 4

Determination of TgMIF structural biology

4.0 Abstract

Elucidation of the MIF crystal structure in 1996 revealed a trimeric architecture for human and rat MIF proteins (Sugimoto *et al.,* 1996, Sun *et al.,* 1996). Furthermore, structural analysis not only revealed homology of these proteins with bacterial isomerases but in addition provided an insight into the mechanisms by which MIF mediates its catalytic activities. The crystal structure of the previously uncharacterised *T. gondii* homologue of MIF was found to conform to the same global trimeric topology as that of human MIF. In addition, the structure highlighted substitutions in and around the TgMIF tautomerase active site that would account for reduced tautomerase activities. ISO-1 also failed to inhibit TgMIF tautomerase activity , probably as a direct result of these substitutions.

4.1 Introduction

Despite its discovery in 1966, MIF was not successfully cloned until 1989 (Weiser *et al.,* 1989), and it was not until 1996 that the MIF 3D crystal structure was finally solved (Sugimoto *et al.,* 1996, Sun *et al.,* 1996). MIF was shown to exist naturally as a trimer whereby each 12.5KDa monomer consisted of a four stranded beta sheet with two alpha helices vertically arranged (Sun *et al.,* 1996). Determination of the MIF 3D structure also highlighted great homology of the MIF trimer with two bacterial isomerases: 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) and 4-OT whose catalytic activities were also centred around an N-terminal proline (Hajipour *et al.,* 1993, Sugimoto *et al.,* 1996, Bendrat *et al.,*1997). Subsequently, crystal structure studies have proven useful in not only providing insight into the structural architecture of MIF but also detailed analysis of enzyme active sites have aided further understanding of how MIF mediates its catalytic activities (Crichlow *et al.,* 2009).

In the present study, the crystal structure of TgMIF has been elucidated which has allowed a comparative study of this homologue with its human counterpart to be carried out.

4.2 Materials and Methods

4.2.1 Sequence alignments

MIF sequences were acquired from NCBI (http://www.ncbi.nlm. nih.gov/sites/entrez) and sequence alignments were performed using Tcoffee. ESpript (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) was then used to assemble Figure 1a.

4.2.2 Crystallisation

Crystals were grown by the hanging drop, vapour diffusion method in 24-well Limbro plates. Crystals of TgMIF grew at 277 K over a well solution containing 1.82 M ammonium sulphate and 100 mM Tris pH6.5. The drop contained 2 μL of protein solution at 15 mg/mL in 50 mM Tris pH7.5 plus 2 μL of well solution. Prior to data collection, crystals were briefly immersed in a cryoprotectant solution containing 20% (v/v) glycerol plus well solution and then flash frozen in liquid nitrogen.

4.2.3 X-ray data collection and structure determination

All diffraction data were collected at 100K on beam-line I03 at the Diamond Light Source (Oxfordshire, UK), indexed with iMosflm and merged and scaled with SCALA (Anonymous, 1994). For molecular replacement a homology model of the TgMIF structure was generated using the SwissModel server (http://swissmodel.expasy.org/) and used as the search model in PHASER (Anonymous, 1994). Structure building and refinement was performed using COOT (Emsley and Cowtan, 2004) and Refmac, respectively. Data collection and refinement statistics are shown in the appendix (Appendix F).

4.2.4 Tautomerase assays

Tautomerase assays were carried out as detailed in chapter 3.2.

4.2.5 Inhibition assays

80µg TgMIF/0.5µg HsMIF was preincubated with the desired concentration of (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1) (Cambrex) for 30minutes before addition to the normal tautomerase reaction outlined in chapter 2.2.

4.2.6 Statistical Analysis

Data was interpreted using GraphPad Prism Version 5.0 where all data is shown as a mean ± SE where n=3. Significant differences were determined using students t-test where $p<0.05$ was deemed significant.

4.3 Results

4.3.1 TgMIF is predicted to have similar structural architecture to HsMIF

Analysis of sequences was performed using ESprit. TgMIF is predicted overall to have the same global topology as HsMIF with some minor differences being observed. The absence of an η1 turn and a longer α1 helix are predicted in TgMIF by ESpript (Figure 4.1A).

4.3.2 Crystal structure of monomeric TgMIF

The crystal structure of C-terminally His tagged TgMIF was determined to 1.8Å resolution. TgMIF monomer (green) can be superimposed with HsMIF monomer with an r.m.s.d of 1.16Å for all Cα atoms. The global topology between these two monomers is well conserved with the TgMIF monomer consisting of a 4 strand β sheet with two alpha helices vertically arranged. Significantly, the differences predicted by ESpript as previously mentioned (Figure 4.1A) are in fact observed in the TgMIF monomer structure (Figure 4.1B).

4.3.3 TgMIF exists as a trimer

TgMIF adopts a trimeric ring architecture with the formation of a central hydrophilic pore (Figure 4.2A). This structure is in keeping with published data from other known MIFs (Figure 4.2B). Superimposition of TgMIF (green) with HsMIF (blue) reiterates structural conservation of the trimer in TgMIF (Figure 4.2C).

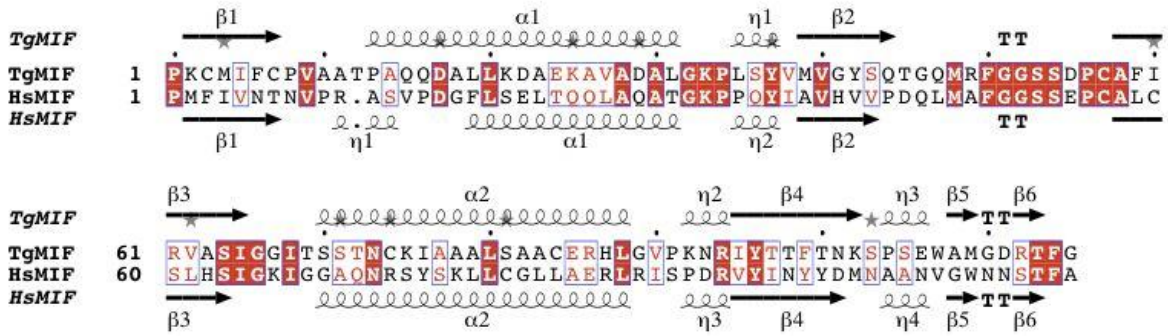
4.3.4 Sulphate ions are found in the TgMIF pore

The formation of a trimer results in a narrow pore in TgMIF in which sulphate ions are located. Detailed structural analysis reveals potential interactions for these ions which are uncharacterised in any other MIF proteins to date (Figure 4.3)

4.3.5 Detailed analysis of the tautomerase active site explains reduced catalytic activity of TgMIF

Analysis of TgMIF structure reveals critical amino acid substitutions in and around the tautomerase active site. Substitution in the active site of an asparagine for a threonine at position 98 in TgMIF is observed in addition to the further substitution of a valine[106] for a tryptophan[107] around the active site area (Figure 4.4A).

A



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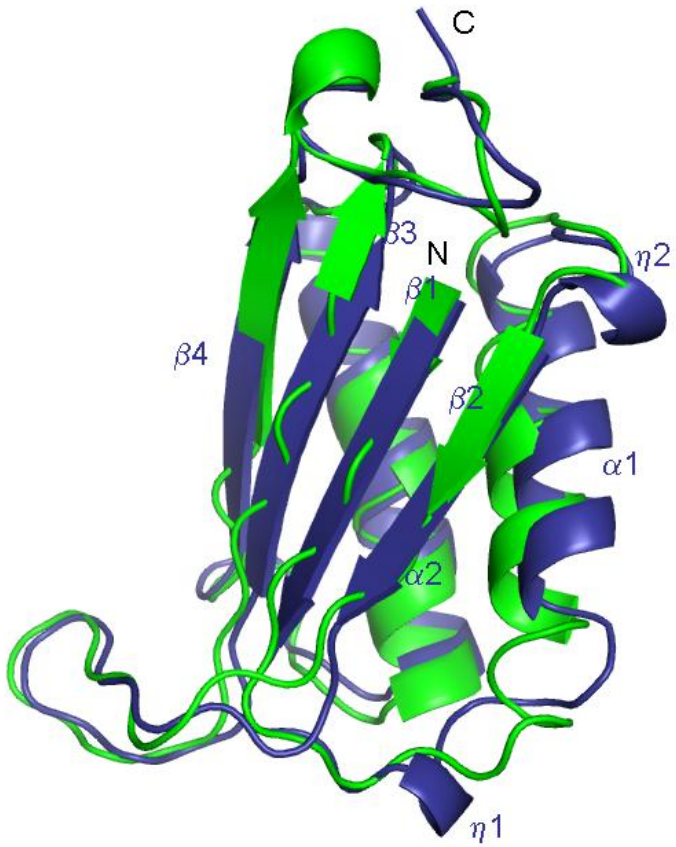


Figure 4.1: Comparison of TgMIF and HsMIF

Secondary structure features are found above and below alignment for TgMIF and HsMIF respectively (Figure 4.1A). Superimposition of chain A of HsMIF (Blue) superimposed with chain A of TgMIF. R.M.S.D is 1.16Å over all carbon atoms (Figure 4.1B).

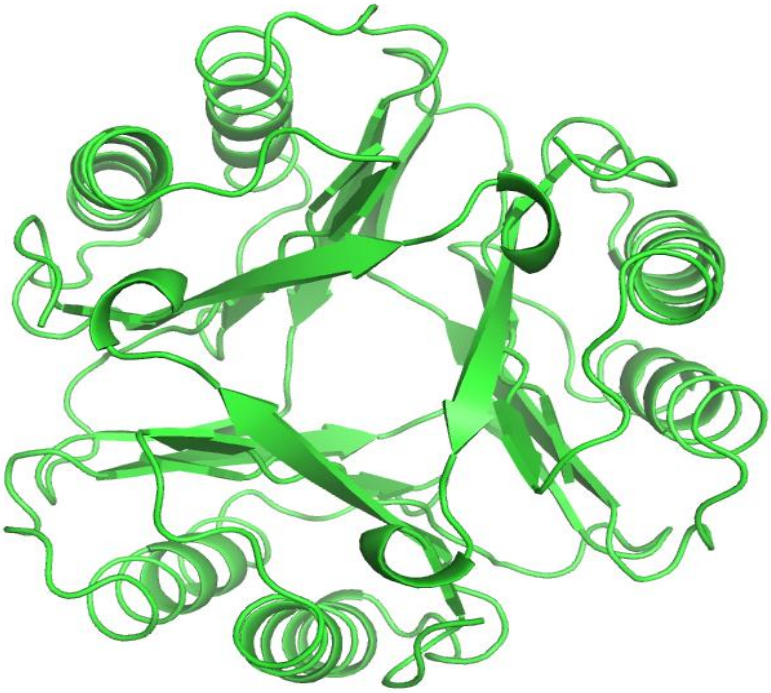
Superimposition of structures shows where ISO-1 (orange) would bind MIF trimers (Figure 4.4B). In addition, the presence of three sulphate atoms bound in the pore of the TgMIF trimer are evident from this representation.

Detailed analysis of the tautomerase active site of both HsMIF (blue) and TgMIF (green) in complex with ISO-1 (orange) illustrates a successful interaction between
HsMIF and ISO-1. However, access of ISO-1 to the TgMIF active site is restricted by Trp^{107}(Figure 4.4C).

4.3.6 TgMIF tautomerase activity is not inhibited by ISO-1

In order to determine whether or not TgMIF tautomerase activity was relatively unaffected by ISO-1 as predicted by structural analysis, TgMIF was pre-incubated with a variety of concentrations of ISO-1 (Figure 4.4D). A weak dose response was observed following preincubation of TgMIF with ISO-1 across a variety of concentrations ranging from 2.5µg to 40µg. Inhibition ranged from 0% to 9.9% across the concentration gradient with 3.5% inhibition being measured with 10µM ISO-1. This is in contrast to HsMIF, which was inhibited by ~60% in the presence of 10µM ISO-1(Figure 4.4E).

A



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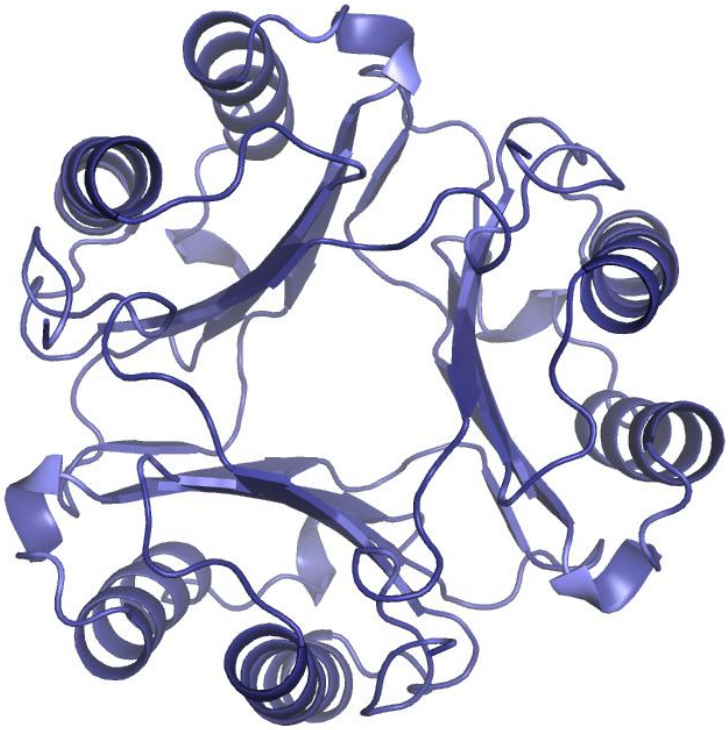

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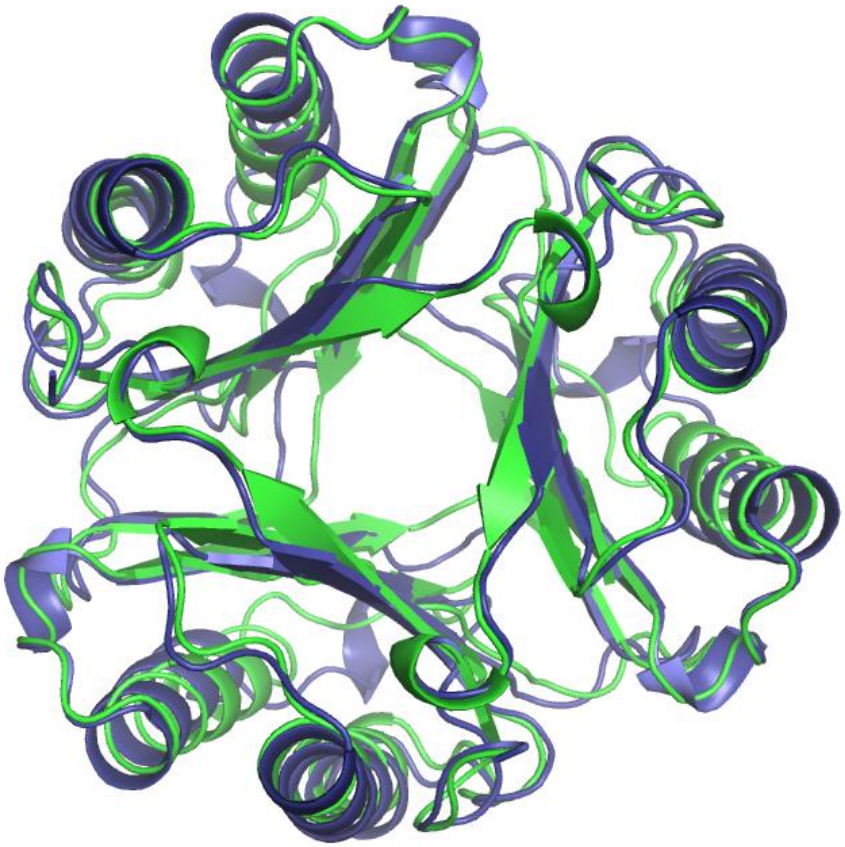


Figure 4.2: TgMIF has a trimeric architecture

Ribbon diagram of TgMIF trimer is shown (Figure 4.2A) as is that of HsMIF (Figure 4.2B). Trimers of two MIFs are superimposed where human MIF is represented in blue and TgMIF is represented in green (Figure 4.2C).

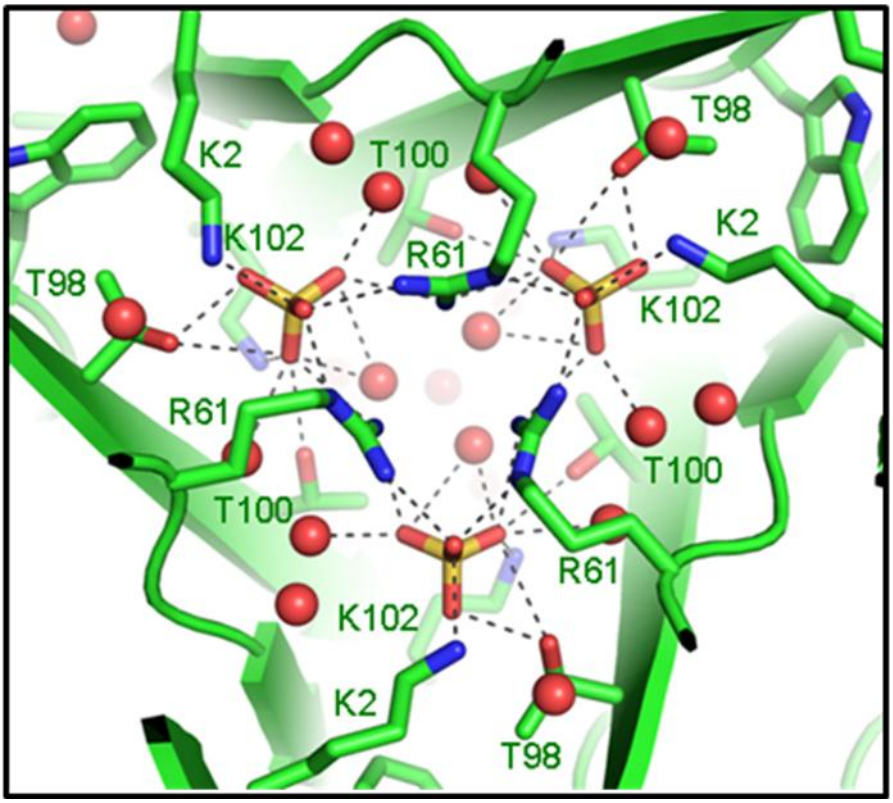


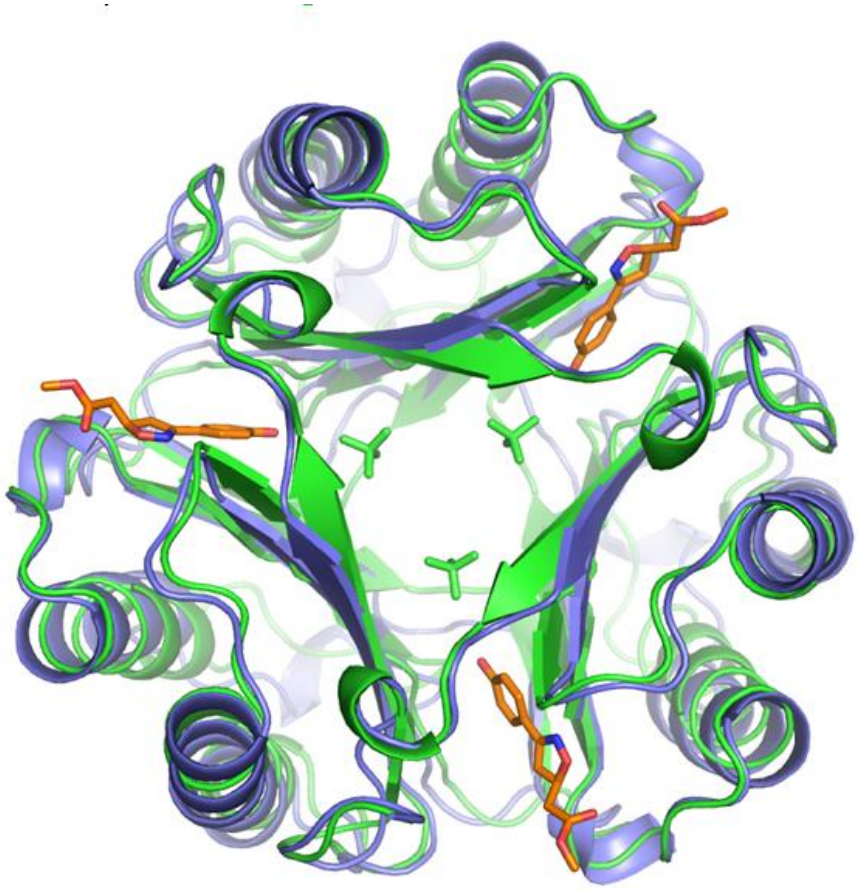
Figure 4.3 Detailed analysis of sulphate ions in TgMIF pore

Unique to TgMIF, sulphate ions are found located within the pore. Potential interactions between sulphate ions in the TgMIF pore with surrounding positively charged amino acids are highlighted above.

A

HsMIF	**TgMIF**
P1	P1
K32	K33
I64	I65
Y95	Y96
N97	T98

B



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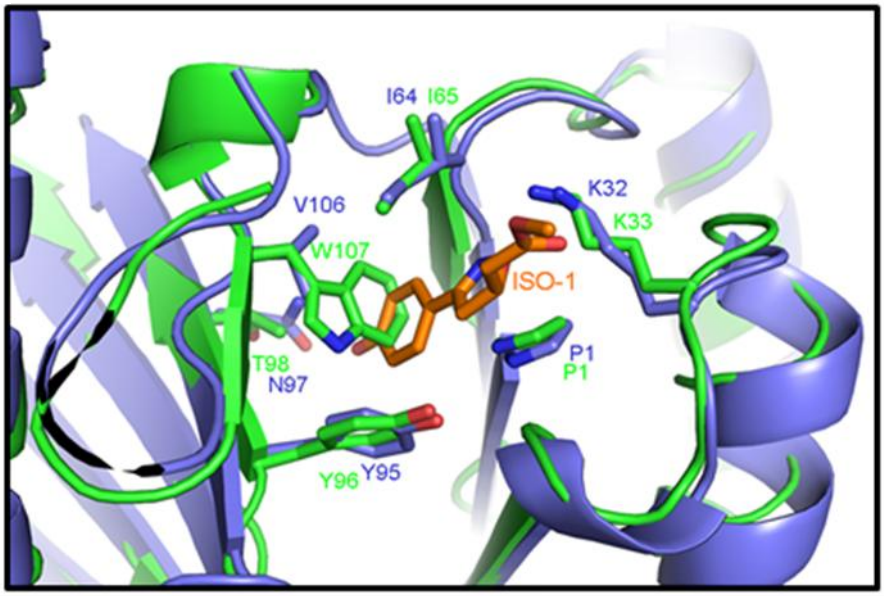

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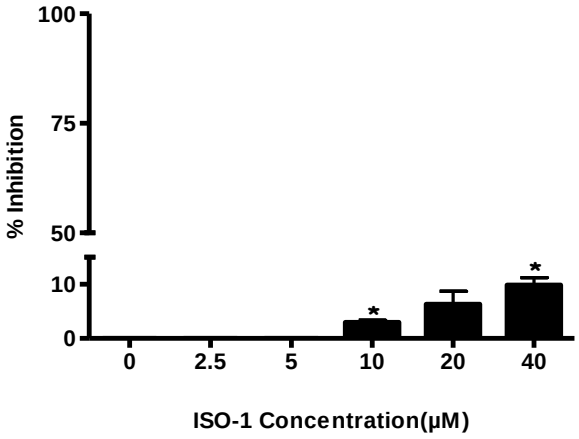

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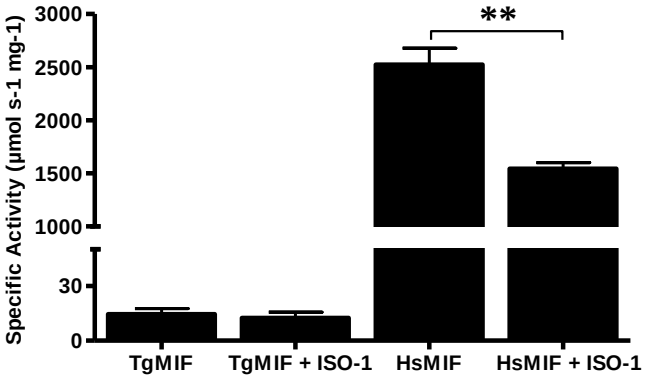


Figure 4.4: Structural analysis of tautomerase active site

Comparison of differences in the active site residues between HsMIF and TgMIF (Figure 4.4A). HsMIF (blue) is superimposed with TgMIF (green) and complexed with active site specific inhibitor ISO-1 (orange) (Figure 4.4B). Detailed analysis of the active site highlights amino acid differences and problems with steric hindrance (Figure 4.4C). Inhibitory effects of ISO-1 on TgMIF tautomerase activity are investigated via pre-incubation of TgMIF with increasing concentrations of ISO-1 (Figure 4.4D). Comparison of HsMIF and TgMIF tautomerase activity following pre-incubation with 10μM ISO-1 (Figure 4.4E).

4.4 Discussion

Determination of the structural properties of a molecule can often provide essential information regarding potential functions for the structure in question. In this study, the crystal structure of TgMIF was determined and comparative studies were carried out between this homologue and its human counterpart.

Secondary structure analysis of TgMIF reveals the absence of a short η1 turn which is evident in mammalian MIFs. In addition, a conserved proline at position 15 is substituted for a glutamine in TgMIF, an amino acid which usually functions as a 'breaker' residue between the η1 turn and α1 helix (Richardson *et al.*, 2009). Furthermore, a second region of structural difference was observed in the length of the α1 helix which expands from residues 13 to 31 in TgMIF resulting in an alpha helix which is significantly longer than that observed in human MIF. Interestingly, these structural differences are mirrored exactly in LmMIF (Richardson *et al.*, 2009).

Analysis of structural data shows TgMIF exists as a trimer whereby each monomer consists of a four stranded mixed beta sheet and two alpha helices stacked against a beta sheet. The formation of a trimer results in a topology which is greatly conserved with HsMIF as seen in superimposition images. Of interest, the TgMIF trimer appears to form a hydrophobic pore which is narrower than that of HsMIF. The HsMIF pore is described as a unique solvent channel which in combination with its positive electrostatic field has been suggested to be involved in transportation (Sun *et al.*, 1996). However, to date, the pore has no confirmed function and therefore it is difficult to determine the biological consequences as a result of differences in pore diameter.

Conservation of global topology of MIF within homologues is not uncommon (Suzuki *et al.*, 1996, Suzuki *et al.*, 2004). In fact, to date, all of the crystal structures of parasite MIF homologues have generally retained the overall structure of mammalian MIF, both in the monomer and trimer forms (Tan *et al.*, 2000, Cho *et al.*, 2007, Kamir *et al.*, 2008). Differences observed are generally at the amino acid level which can often result in small structural variations, although, these differences generally do not greatly modify the overall protein conformation.

Structural analysis reveals amino acid substitutions in the area of the tautomerase active site which could ultimately alter the catalytic activity of TgMIF. Tautomerase activity involves the Pro^{1}, Lys^{32} and Ile^{64} of one monomer in an interaction with Tyr^{95} and Asn^{97} of the adjacent monomer (Lubetsky *et al.*, 1999). Most amino acids of the active site are conserved in addition to Tyr^{36} and Phe^{114} which surround the active site. However, replacement of an Asn^{98} for a Thr in the TgMIF tautomerase active site is of importance as this substitution prevents the formation of a hydrogen bond between MIF and L.DOPA methyl ester (Richardson *et al.*, 2009). An identical substitution has previously been reported in *A. ceylanicum* MIF with the suggestion that these substitutions may account for the weakened tautomerase activities (Cho *et al.*, 2007). With this in mind, there is also a strong possibility that this substitution is likely to be responsible for the great reduction in catalytic activity observed in TgMIF for L.DOPA methyl ester compared with HsMIF.

Further substitutions around the TgMIF tautomerase active site are observed whereby Val^{106} is replaced by Trp^{107} resulting in steric hindrance which could prevent the successful binding of ISO-1 to the active site. Concentrations of ISO-1 were selected on the basis of a previous study by Lubetsky *et al.*, (2002) whereby HsMIF was shown to have an IC50 of ~7µM. Pre-incubation of HsMIF and TgMIF with 10µM ISO-1 allowed a comparative study to be performed. HsMIF tautomerase activity was inhibited by ~60% following pre-incubation with 10µM ISO-1, which is similar to previously published results (Lubetsky *et al.*, 2002). Further investigation into the potential inhibitory effect of ISO-1 on TgMIF tautomerase activity revealed a very slight dose response to ISO-1. However, in comparison to HsMIF, the effects of ISO-1 on TgMIF tautomerase activity are significantly reduced and almost negligible when compared with HsMIF IC50. These results for TgMIF are similar to that observed in other parasite homologues of MIF (Kamir *et al.*, 2008, Cho *et al.*, 2007)

In addition to ISO-1, inhibition studies of tautomerase activity have also been performed using dopachrome analogs (Zhang and Bucala, 1999) and other inhibitors (Senter *et al.*, 2001). However, to date there have been limited studies investigating the effects of inhibitors of parasite homologues of MIF. However, Kamir *et al.*, (2008) demonstrated that like TgMIF, ISO-1 selectively inhibited HsMIF tautomerase activity but had no significant effect on LmMIF activity. Further inhibition studies with 4-iodophenylpyrimidine (4-IPP) resulted in complete inhibition of tautomerase activity being observed in both HsMIF and LmMIF, by covalent modification of the N-terminal proline. 4-IPP also inhibited LmMIF induced

migration of monocytes, an effect which was not observed with non inhibitory ISO-1. This would suggest that the tautomerase activity of LmMIF is directly related to modulation of macrophage migration which would have significant implications for survival of this parasite in the host (Kamir *et al.,* 2008). Furthermore, increasing concentrations of 4-IPP resulted in a dose dependent reduction in MIF and p115 secretion from LPS induced THP-1 macrophages (Merk *et al.,* 2009). Consequently, 4-IPP modification of MIF induces p115 binding to MIF, which the authors suggest inhibits MIF release from cells by interfering with additional protein interactions necessary for secretion (Merk *et al.,* 2009). Therefore, it would be desirable to determine the inhibitory effects of other substances on TgMIF tautomerase activity which might then be used in further studies to determine TgMIF function.

Of particular interest, structural analysis reveals the presence of many sulphate ions within the TgMIF pore, a feature which has previously been unreported in other MIFs. The negatively charged sulphate ions are able to interact with the positively charged side chains of Arg^{61}, Lys^{2} and Lys^{102} in a series of links which is likely to act to stabilise the interface of the trimer in the pore. Also of interest is an interaction between sulphur ions and Thr^{98}, an active site residue. This indicates a potential role for the pore in tautomerase activity or alternatively, the sulphate ions may act to mimic a phosphate binding site. The latter may therefore suggest phosphorylation on a binding partner protein that binds in the pore with its phosphate in the position (Yun-Jeong *et al.,* 2003, Dessailly *et al.,* 2007).

Chapter 5

Characterisation of TgMIF immunological properties

5.0 Abstract

It has been suggested that many of the pro-inflammatory activities attributed to MIF may be as a consequence of endotoxin contamination. Furthermore, it has also been suggested that ERK-MAPK activation and IL-8 production are the main indicators of MIF activity. In the process of this study, recombinant LPS-free TgMIF was shown to be capable of inducing IL-8 production from human PBMC. Furthermore, TgMIF is capable of inducing ERK-MAPK activation in murine bone marrow-derived macrophages whilst having no significant effect on CD74 or CD44 expression. Finally, in preliminary experiments, a decrease in TLR-4 mRNA levels has been observed following stimulation with both TgMIF and LPS.

5.1 Introduction

Identification of MIF as a secreted mediator which inhibited the random migration of macrophages (Bloom and Bennett, 1966, David, 1966) subsequently led to multiple studies attempting to define other biological functions of MIF, and indeed, how these functions were mediated. Consequently, MIF has been attributed with being responsible for many activities including upregulation of TLR-4, augmentation of pro-inflammatory cytokine production, inhibition of immunosuppressive properties of GC and activation of ERK MAPK pathways (Roger *et al.,* 2001, Calandra *et al.,* 1995, Mitchell *et al.,* 1999). However, some have debated the authenticity of these results as several studies failed to document endotoxin levels present in protein preparations or indeed whether an endotoxin removal step was performed (Nguen *et al.,* 2003, Bernhagen *et al.,* 1994, Baumann *et al.,* 2003).

The immunological roles of selected parasite MIF homologues have been partially characterised. Subsequently, most investigations involving parasite MIF homologues have focused on the ability of these proteins to effect monocyte migration and protein interactions with CD74.

Herein, the successful production of recombinant LPS-free TgMIF has allowed for the partial characterisation of the biological functions of TgMIF.

5.2 Materials and Methods

5.2.1 Large scale protein expression, purification and estimation

Protein was expressed, purified and quantified as described in 2.2.19, 2.2.20 and 2.2.21

5.2.2 Endotoxin removal

A ProteoSpin® Endotoxin Removal Maxi (Novagen) column was equilibrated with 10ml of column activation and wash buffer by centrifugation at 1000 g for 2 minutes. 4mg TgMIF was diluted in distilled water to 5mls before the addition of 40µl pH binding buffer. The solution was then added to the activated column, to which 50µl endotoxin removal solution was subsequently added. The column was then mixed gently by vortex and incubated at room temperature for 5 minutes after which time 500µl isopropanol was added. Protein was then bound to the column by centrifugation at 1000g for 5 minutes and the flow through was discarded. The centrifugation step was then repeated. Bound protein was then washed by the addition of 10ml column activation and wash buffer and centrifugation for 3 minutes at 1000g. The wash step was then repeated. The column was then spun dry for 2 minutes at 1000g. To elute protein, 140µl neutraliser was added to a clean elution tube and 2ml of elution buffer was added to the column. The column was then centrifuged for 5 minutes at 3000g to elute protein. Following elution, protein was quantified as described 2.2.21.

5.2.3 Chromogenic Endotoxin Test QCL 1000

Endotoxin levels were tested using QCL 1000 endotoxin chromogenic endpoint kit (Cambrex, UK). Endotoxin standards (Cambrex, UK) were prepared ranging from 0.01Eu/ml to 1Eu/ml in endotoxin-free microcentrifuge tubes (Fisher, UK) prior to the addition of 50µl of each standard to pre-equilibrated 96 well tissue culture plate (TPP, Switzerland). 50µl of LAL reagent water, sample buffer and sample at working concentration were also added to wells. To each well, 50µl freshly reconstituted LAL lysate was then added and incubated at 37°C for 10minutes. Following this, 100µl of freshly reconstituted prewarmed chromogenic substrate was added to each well and incubated at 37°C for 6 minutes. To stop the reaction, 100µl 25% glacial acetic acid (Acros organics, Geel, Belgium) was added to each well and the plate was read at 405nm.

5.2.4 Isolation of human peripheral blood mononuclear cells (PBMC)

Human PBMC were isolated from freshly drawn blood using Histopaque (Sigma – Aldrich). To a 15ml centrifuge tube, 3ml of prewarmed HISTOPAQUE-1077 was added on top of which

3ml whole blood was carefully layered. The solution was centrifuged at 4000g for 30 minutes at room temperature to separate the blood into plasma, mononuclear cells and red blood cells. Following centrifugation, the plasma in the top layer was carefully removed and discarded. The opaque interface containing mononuclear cells was then removed and added to a clean 15ml centrifuge tube to which 10ml RPMI1640 (PAA, Pasching, Austria) was added. The tube was then centrifuged for 10minutes at 250g following which the supernatant was removed and the cell pellet was resuspended in 5ml RPMI1640 (PAA, Austria) and spun again at 250g for 10minutes. Following this, the supernatant was removed and the pellet resuspended again in 5ml RPMI1640 (PAA, Austria). The resuspended pellet was centrifuged again at 250g for 10minutes following which the supernatant was removed and the pellet resuspended in 0.5ml cRPMI. Isolated mononuclear cells were then counted and seeded to 96 well plates at $5x10^6$ cells/ml in RPMI1640 containing 10% heat-inactivated fetal calf serum, 2mM L-Glutamine, 100U/ml penicillin and 100µg/ml streptomycin at 37°C and 5% CO_2. Cells were stimulated with increasing concentrations of endotoxin free TgMIF (1ng/ml-100ng/ml) for 24 hours.

5.2.5 IL-8 ELISA

In order to detect IL-8 concentrations in cell supernatants, 96 well plates were coated with 50µl/well purified mouse anti-human IL-8 (BD PharMingen) at 2µg/ml in PBS pH9 and incubated overnight at 4°C. Plates were then washed three times in PBS pH 7.4 with 0.05% Tween 20. Wells were then blocked by the addition of 200µl/well blocking buffer (10% fetal calf serum in PBS pH7.4) and incubated at 37°C for one hour. Plates were then washed three times before the addition of 30µl supernatants to appropriate wells. Recombinant human IL-8 was also added to corresponding wells in doubling dilutions ranging from 20ng/ml to 387.5pg/ml and plates were then incubated at 37°C for two hours. Plates were then washed four times prior to the addition of 50µl/well 1µg/ml biotinylated anti human IL-8 (BD PharMingen) and then incubated for a further hour at 37°C. Streptavidin alkaline phosphatase (Pharmingen) diluted 1:2000 was then added to the plates, 50µl/well, following a previous washing step and incubated for 45 minutes at 37°C. Following a final wash step, 100µl p-nitrophenyl phosphate (Sigma) at 1mg/ml in glycine buffer was added to plates. Absorbances were recorded at 450nm using SPECTRAmax 190 microtitre plate spectrophotometer. All assays were carried out in triplicate.

5.2.6 Isolation of macrophages from bone marrow stem cells

Femurs were removed from male BALB/c mice and flushed using a 25 gauge needle with 5ml macrophage medium. Macrophage media consists of 30% (15ml) L-cell conditioned supernatant, 20% (10ml) heat-inactivated fetal calf serum, (0.5ml) 2mM L-Glutamine, (0.5ml) 100U/ml penicillin and 100µg/ml streptomycin in Dulbeccos Modified Eagles medium supplemented with sodium pyruvate, glucose and pyridoxine hydrochloride (Invitrogen, UK). Flushed bone marrow stem cells were collected in a 50ml tube. Cells were then passed through a 21 gauge needle before being split between petri dishes. Each petri dish was then supplemented with an additional 10mls macrophage medium and cells were incubated at 37°C/ 5% CO_2. Cultures were then supplemented with 10mls macrophage medium on day 3. On day 7, medium was completely removed from petri dishes to discard non adherent cells and 20mls fresh macrophage medium was added to each petri dish. Confluent macrophages were harvested on day 10 by the removal of macrophage medium and replacement with 5mls cold RPMI1640. Cells were then removed using a 24cm cell scraper (TPP, Trasadingen, Switzerland) and spun at 300g for 5 minutes. The supernatant was removed by aspiration and the pellet resuspended in 10ml macrophage media. Cells were then washed by centrifugation at 300g for 5 minutes. The supernatant was removed and the cells were washed again in 10ml complete RPMI (cRPMI) (Invitrogen) by centrifugation at 300g for 5 minutes. cRPMI consisted of RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, 2mM L-Glutamine, 100U/ml penicillin and 100µg/ml streptomycin The supernatant was removed and the cell pellet was resuspended in 2ml cRPMI. A dilution of the cell suspension was made in 0.4% trypan blue (Sigma-Aldrich) and cells were counted using a haemocytometer. Cells were then diluted to the desired concentration.

5.2.7 Stimulation of macrophages for real time PCR

Cells were seeded in a 24 well plate (TPP) $2x10^6$ cells/ml in a total volume of 500µl. Cells were then incubated overnight at 37°C/ 5% CO_2 to allow macrophages to adhere. Following this, cells were stimulated with increasing concentrations of TgMIF (1ng/ml-5µg/ml) or 1ng/ml recombinant human MIF (R&D Systems). For experiments to determine TLR-4 expression, cells were supplemented with 100ng/ml TgMIF and 200ng/ml LPS from *E. coli* 055:B5 (Sigma-Aldrich, UK). Cells to assess CD74/CD44 expression were incubated for 4 hours whereas experiments to assess TLR-4 expression were incubated for the durations indicated in the time course. All cells were incubated at 37°C/ 5% CO_2.

5.2.8 Sample preparation, RNA extraction and cDNA synthesis for real time PCR

Supernatants were removed from wells and 4 x 10^6 cells were collected in 1ml Trizol per treatment. Samples were then collected in a 2ml DNase/RNase free screw cap tube (Starlab, UK). RNA was extracted from samples as detailed in 2.2.3. Samples were screened for gDNA contamination by PCR as described 2.2.4 and cDNA was synthesised from clean RNA as described in 2.2.5.

5.2.9 Preparation of standards for real time PCR

Standards were prepared for CD74, CD44, TLR-4 and TBP. The genes of interest were amplified using specifically designed primers (see appendix) by PCR as described 2.2.6. Amplified gene portions were excised from the agarose gel and DNA was isolated from the agarose by gel extraction as described 2.2.7. In a ligation reaction, 2µl purified PCR product was inserted into 0.5µl pDRIVE (Qiagen, UK) with 2.5µl ligation buffer (Qiagen, UK) overnight at 4°C. Ligation reactions were transformed as described 2.2.10 and 2.2.11 except agar plates were coated with 100µl 100mM IPTG (Biogene, Hudington, UK), 20µl of 100µg/ml ampicillin (Sigma-Aldrich, UK) and 20µl of 50µg/ml X-gal (Apollo Scientific, Stockport, UK) prior to the addition of 140µl transformed cells. Agar plates were screened the following day for the formation of white colonies. This screening system works on the principle that successful insertion of gene products disrupts the *lacZ* gene resulting in a dysfunctional β-galactosidase which is responsible for the production of blue colonies. Therefore, a white colony was selected, indicative of a plasmid with a successfully inserted PCR product, and inoculated into a 10ml LB broth containing 10µl ampicillin and incubated overnight at 37˚C on a rotary shaker (225rpm). Plasmids were then purified as described 2.2.13. Subsequently plasmids were screened for successful insertion by restriction enzyme digest in a reaction where 5µl plasmid was incubated with 1µl *Eco*RI (Promega, Southampton), 1µl buffer H and 2µl molecular grade water (Sigma-Aldrich) at 37°C for 2 hours. Digest products were visualised on a 1.8% agarose gel. Clones containing successfully inserted DNA were quantified using a spectrophotometer and dilutions were made to produce a range of standards from 3 x 10^6 to 3 x 10^1 copies. Standard curves are shown in appendix D.

5.2.10 Quantitative real time PCR

Real time PCR reactions contained 6.25µl SYBR green (Agilent Technologies, Santa Clara, USA), 5.25µl molecular grade water (Sigma Alrdich), 25pmol forward primer

, 25pmol reverse primer and 1µl template (cDNA from samples or standards). Specific primers were used for the gene of interest to be amplified (see appendix A). For negative controls, 1µl molecular grade water was added instead of cDNA. Reactions obeyed the following thermal profile: 1 cycle at 95°C for 10minutes, 40cycles for 1 minute at 95°C, 1 minute at specific annealing temperature (see appendix) and 1 minute at 72°C. Finally reactions were subjected to 1 minute at 95°C, 30 seconds at 55°C and 30 seconds at 95°C. Copy numbers of the gene of interest present in each sample were automatically determined by the Stratagene MXP3000 software. Relative expression of the gene of interest was then calculated based on comparison of expression of the house-keeping gene *Tbp*.

5.2.11 Stimulation of macrophages for Western blot

Cells were seeded in a 6 well plate (Nunc, Loughborough, UK) at $1x10^6$ cells/ml in a total volume of 1ml. Cells were then incubated overnight at 37°C/ 5% CO_2 to allow macrophages to adhere to wells. Cells were stimulated with TgMIF (100ng/ml) or 1ng/ml recombinant human MIF (R&D Systems) over a time course ranging from 5 minutes to 120 minutes. Cells were incubated at 37°C/ 5% CO_2 throughout the duration of the experiment.

5.2.12 Sample preparation for Western blot

To harvest cells, the medium was removed and cells were washed with 500µl PBS which was subsequently removed by aspiration. To each well, 150µl sample buffer was added and cells were collected by agitation of the well surface with the plunger of a 2ml syringe. Samples were stored in microcentrifuge tubes which were boiled prior to use in Western blot.

5.2.13 ERK MAPK Western blot

An SDS polyacrylamide gel was set as described 2.2.15 and 25µl of each sample was loaded per lane. Western blots were performed as described in 2.2.22. Membranes were probed with (1:7500) anti-phosphorylated ERK and anti-total ERK antibody (Santa Cruz Biotechnologies, Santa Cruz, USA). Membranes were subsequently probed with an HRP linked anti-rabbit IgG or HRP linked anti-mouse IgG secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, USA), for detection of total and phosphorylated ERK respectively. Secondary antibodies were diluted to 1:7500 for use.

Developed blots were scanned using a Epson Perfection 164054 Scanjet using Epson Twain 55.52 Scan Jet picture software. Scanned blots were normalised to control samples (total ERK) and quantified using Scion Image (Scion Corporation, Maryland, USA).

5.2.14 Statistical Analysis

Data was interpreted using GraphPad Prism Version 5.0 where all data is shown as a mean ± SE where n=3. Significant differences were determined using students t-test where ***$p<0.0001$ **$p<0.001$ *$p<0.05$ is significant.

5.3 Results

5.3.1 Production of endotoxin-free TgMIF

Purified recombinant TgMIF was removed of endotoxin using ProteoSpin® endotoxin removal columns and subsequent LPS levels remaining in samples were quantified using chromogenic LAL endpoint assay. Recombinant TgMIF typically contained less than 0.008ng/mg protein of LPS at working concentration. A positive control of a separate contaminated protein which had not been subject to an endotoxin removal procedure, was found to have around 48 times more LPS at TgMIF working concentration. Experimental controls were shown to contain negligible amounts of LPS (Figure 5.1).

5.3.2 Endotoxin-free TgMIF drives IL-8 production by human PBMC

TgMIF was found to induce IL-8 from human PBMC in a dose dependent manner. Furthermore, while 1ng/ml HsMIF induced 22520 pg/ml IL-8 production from PBMC, TgMIF induced 7993 pg/ml IL-8. Consequently, TgMIF is 40% as effective at stimulating IL-8 as equivalent levels of HsMIF from PBMC. Results shown are from one run and representative of the pattern of expression observed in other runs (Figure 5.2).

5.3.3 Endotoxin-free TgMIF induces ERK MAPK activation in macrophages

An increase in ERK MAPK activation was observed in macrophages stimulated with 100ng/ml TgMIF. Increase in ERK MAPK activation was represented by an increase in phosphorylation of p42/p44 ERK up to 30minutes post stimulation and thereafter phosphorylation decreases. After 30minutes stimulation with TgMIF, there is a ~30x increase in ERK MAPK activation compared to unstimulated control (Figure 5.3)

The same pattern of activation was observed following stimulation with 1ng/ml HsMIF whereby phosphorylation peaked after 30 minutes stimulation when phosphorylated ERK increased ~270 x compared to unstimulated cells (Figure 5.4)

5.3.4 Endotoxin-free TgMIF has no effect on CD74/CD44 expression on macrophages, except at high concentrations

Following stimulation of bone marrow-derived macrophages with 5μg TgMIF a significant increase in CD74 expression was observed, compared with unstimulated controls. At all other concentrations tested, no significant effect on CD74 expression was observed (Figure 5.5A).

Stimulation of macrophages with TgMIF did not result in a significant increase or decrease in CD44 expression at any of the concentrations tested (Figure 5.5B).

5.3.5 Endotoxin-free TgMIF, in concert with LPS, downregulates TLR-4 expression on macrophages

Stimulation of bone marrow-derived macrophages with 100ng/ml TgMIF had no significant effect on TLR-4 expression compared with unstimulated controls. By comparison, LPS stimulation alone induced significant upregulation of TLR-4 expression. Indeed, TLR-4 expression levels in stimulated cells were equal to those of the unstimulated controls. However, treatment of cells with TgMIF and LPS in combination resulted in an ablation of the LPS-induced upregulation of TLR-4 expression (Figure 5.6).

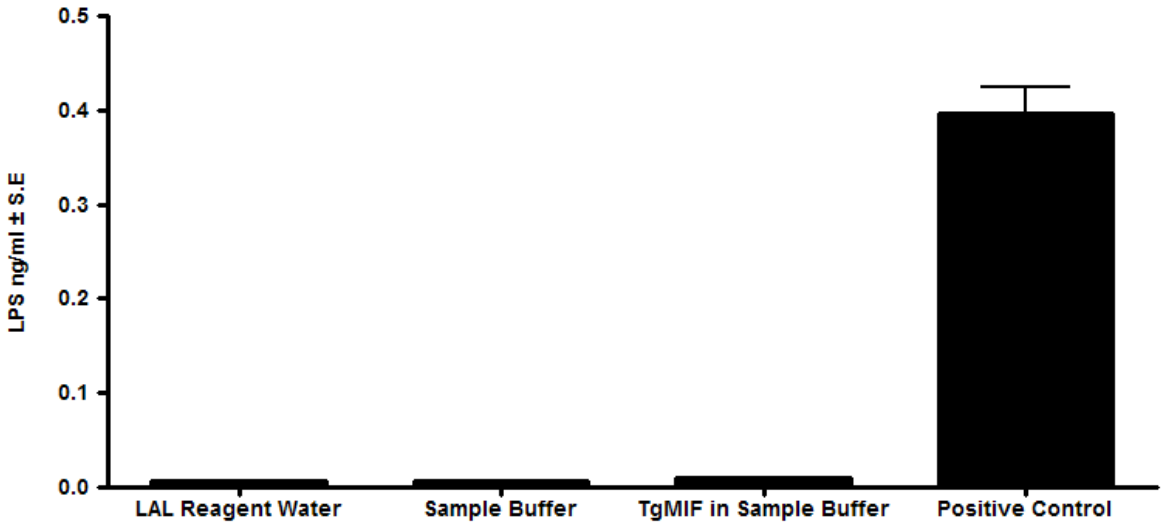


Figure 5.1: Determination of endotoxin contaminants in protein samples.

Protein samples tested via limulus amoebocyte lysate assay prior to stimulation of cells. All LAL assay reagents are also tested for LPS contamination. Protein samples with endotoxin levels <0.01ng/ml were used in stimulations. Positive control: contaminated sample.

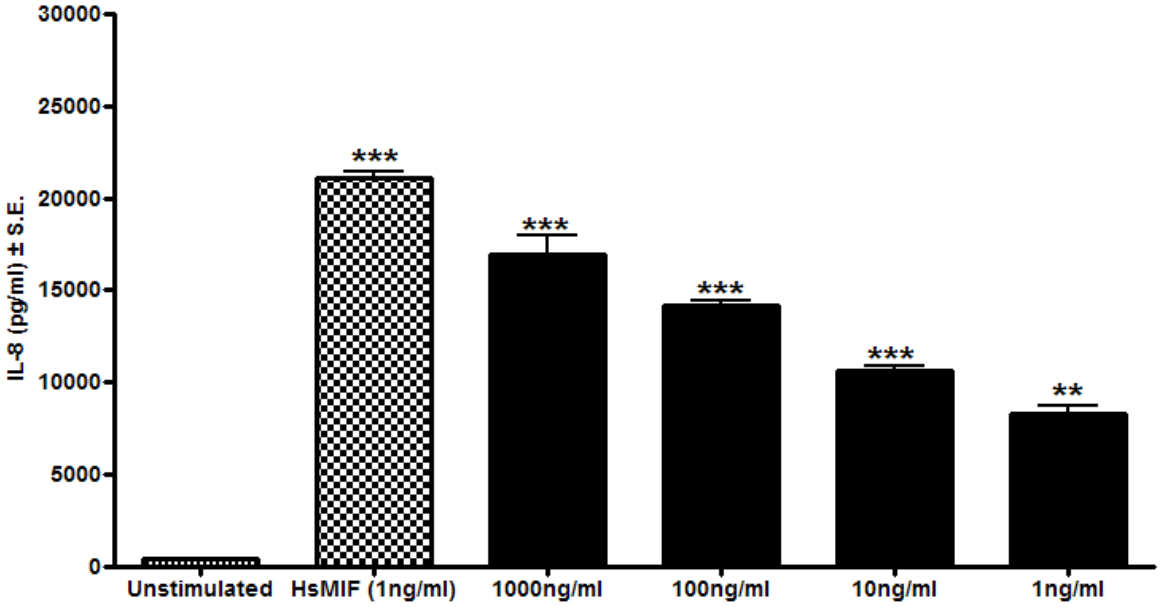
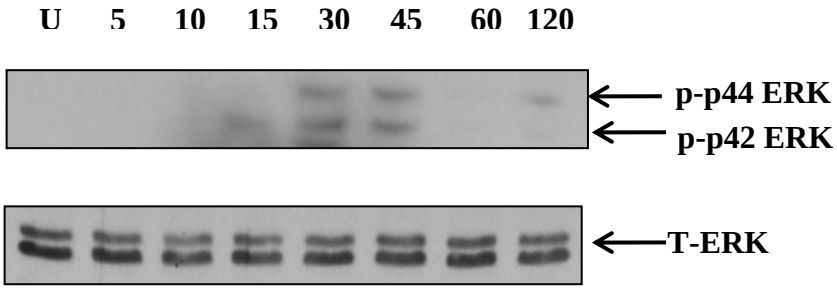


Figure 5.2 The effect of TgMIF on IL-8 production from human PBMC.

The effect of TgMIF on IL-8 production from human PBMC was measured by ELISA. TgMIF stimulation significantly increases IL-8 production from human PBMC in a dose-dependent manner. ***p<0.0001 **p<0.001

A



B

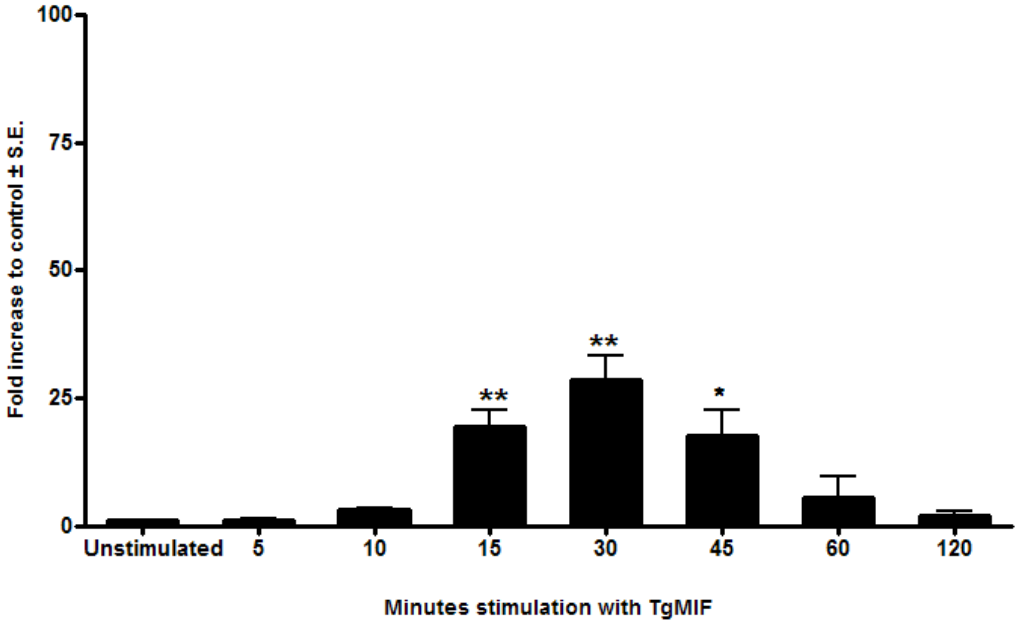
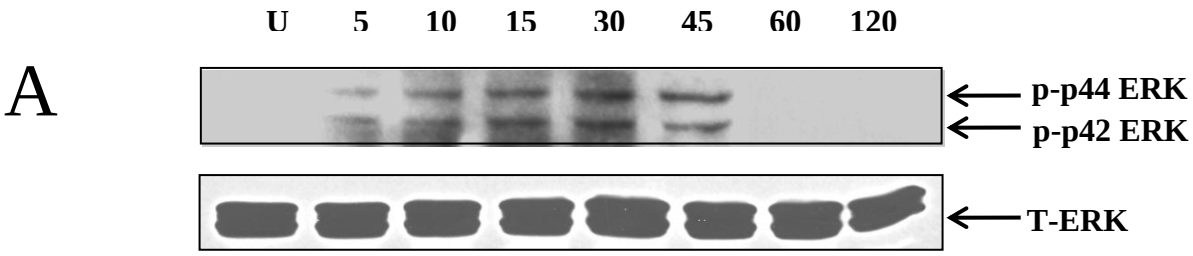


Figure 5.3 The effect of TgMIF stimulation on ERK MAPK activation in macrophages

Stimulation of bone marrow-derived macrophages with 100ng/ml TgMIF results in activation of ERK MAPK which peaks around 30 minutes post stimulation (Figure 5.3A and figure 5.3B). Panel A shows western blots of samples probed for phosphorylated isoforms of p42/44 ERK (i.e. p-p42/44 ERK) as well as total ERK (T-ERK). Scanned blots in Panel A were normalised and quantified by scanning densitometry. Normalised data is shown in Panel B. *p<0.05 **p<0.001



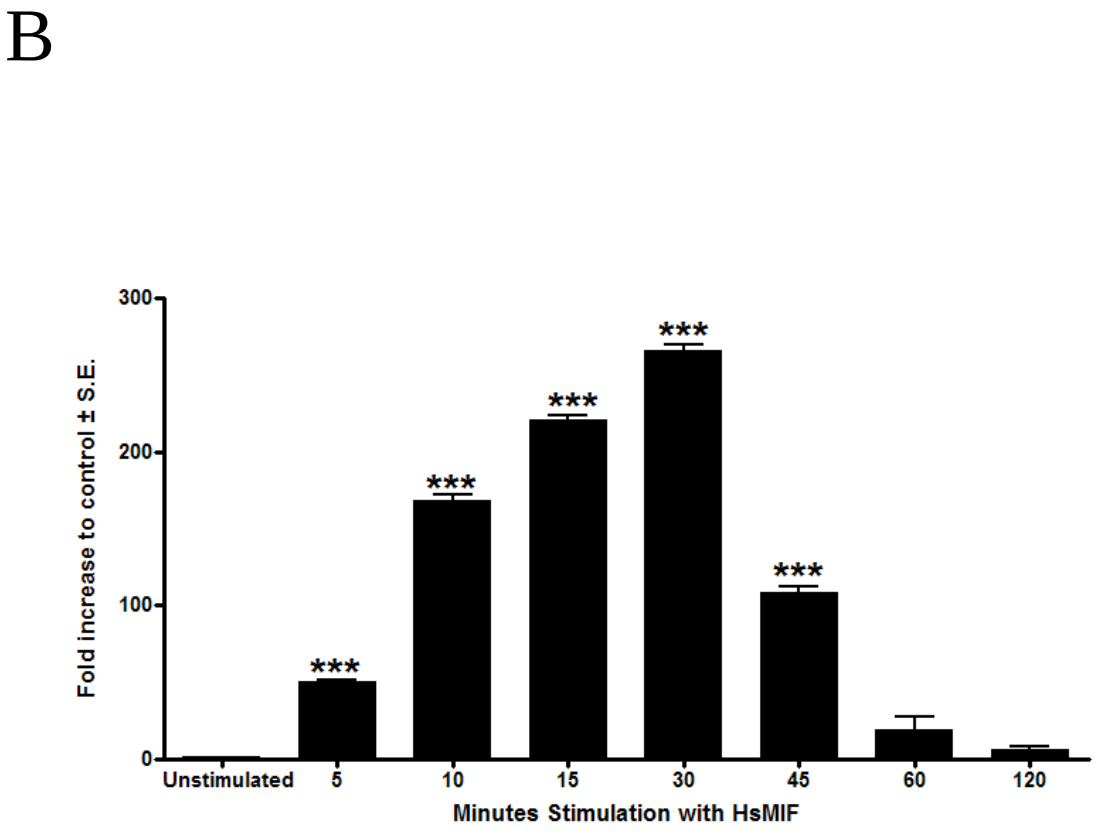
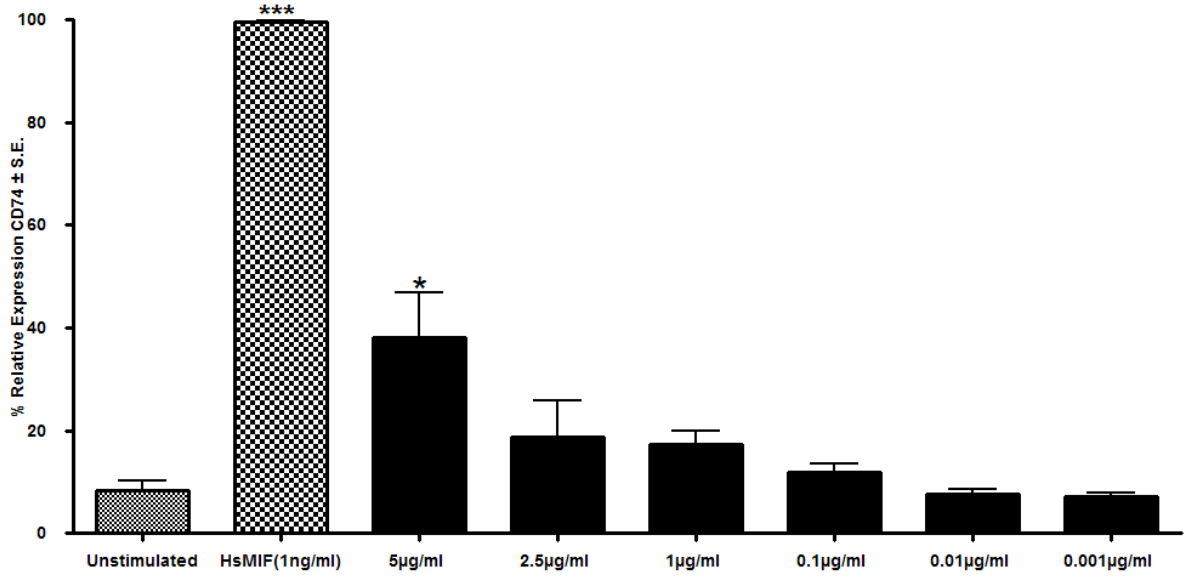


Figure 5.4 The effect of HsMIF on ERK MAPK activation in macrophages

Stimulation of macrophages with 1ng/ml HsMIF results in ERK MAPK activation which peaks around 30 minutes post stimulation (Figure 5.4A and Figure 5.4B). Panel A shows western blots of samples probed for phosphorylated isoforms of p42/44 ERK (i.e. p-p42/44 ERK) as well as total ERK (T-ERK). Scanned blots in Panel A were normalised and quantified by scanning densitometry. Normalised data is shown in Panel B. ***p<0.0001

A



B

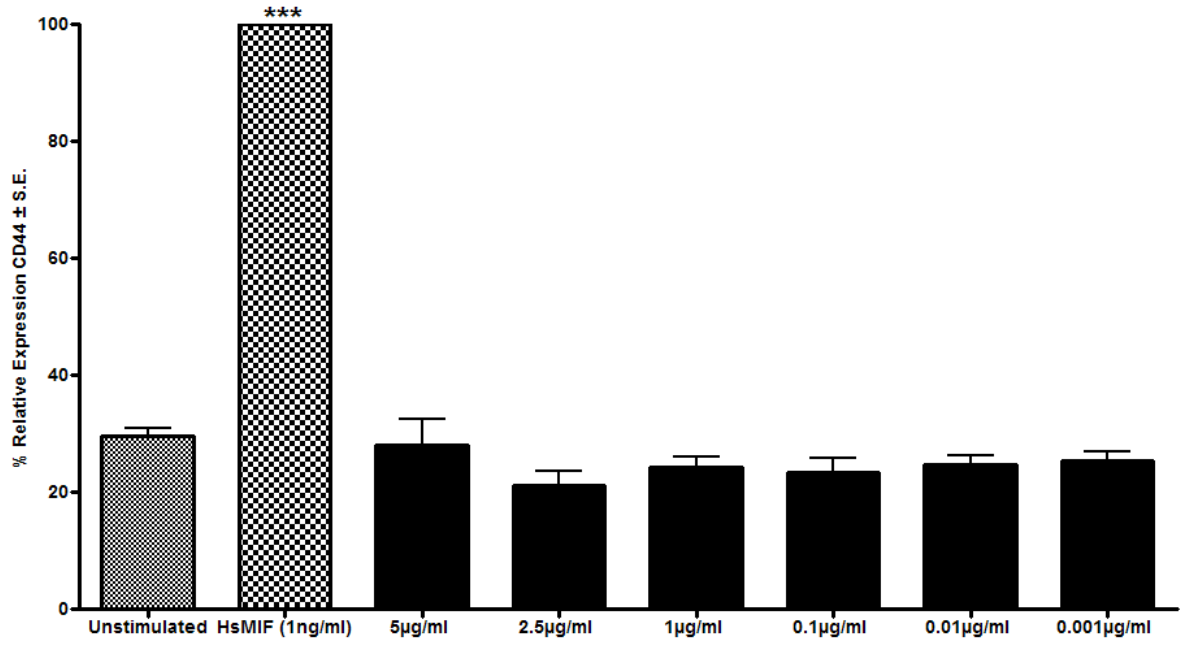
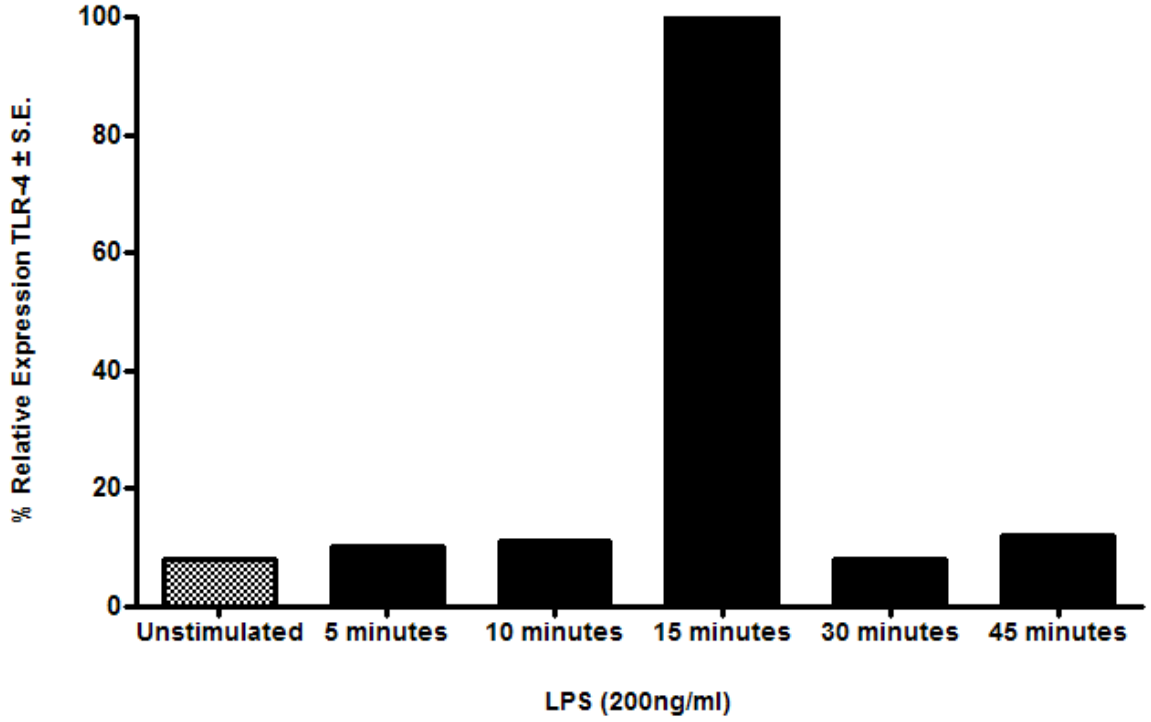


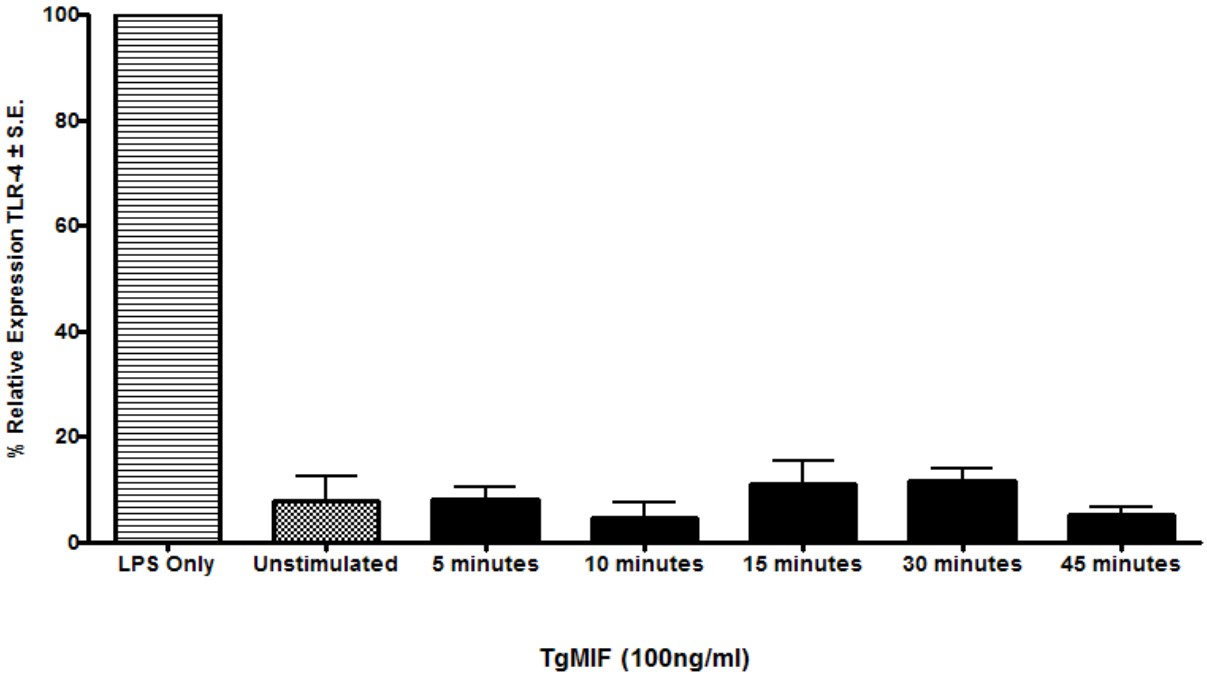
Figure 5.5 The effect of TgMIF on CD74 and CD44 mRNA levels in macrophages

Expression of CD74 and CD44 at the mRNA level was quantified via real time PCR. Macrophages stimulated with increasing concentrations of TgMIF did not significantly increase CD74 expression, except at 5µg/ml (Figure 5.5A). Stimulation with TgMIF did not significantly effect CD44 expression at any of the concentrations tested (Figure 5.5B). . ***p<0.0001 *p<0.05

A


% Relative Expression TLR-4 ± S.E.
100
80
60
40
20
0
Unstimulated
5 minutes
10 minutes
15 minutes
30 minutes
45 minutes
LPS (200ng/ml)

B


% Relative Expression TLR-4 ± S.E.
100
80
60
40
20
0
LPS Only
Unstimulated
5 minutes
10 minutes
15 minutes
30 minutes
45 minutes
TgMIF (100ng/ml)

C

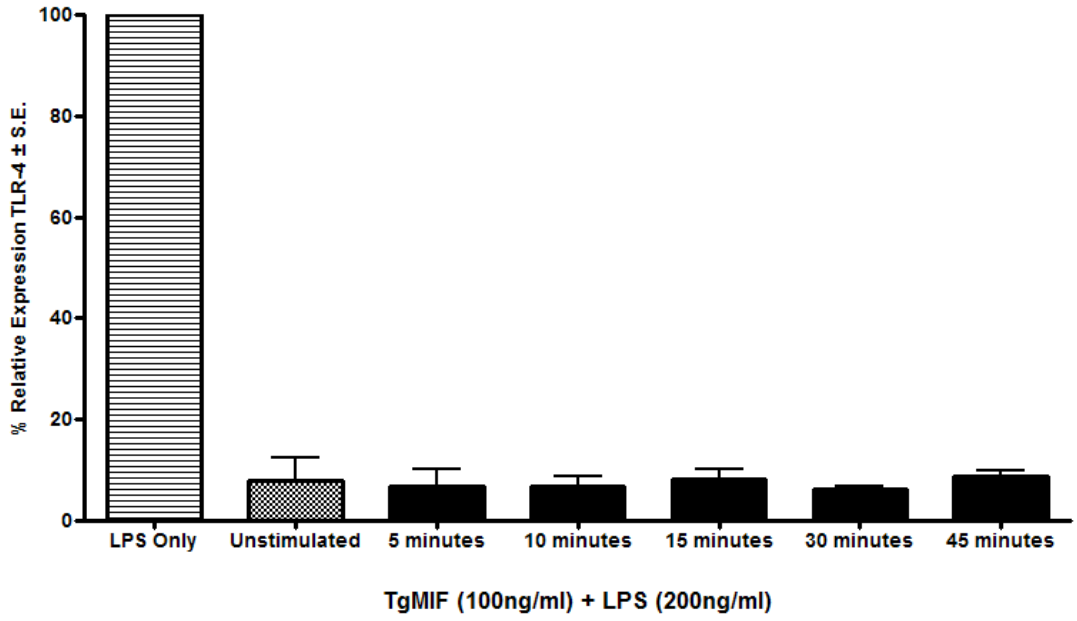


Figure 5.6 The effect of LPS and TgMIF on TLR-4 expression

Expression of TLR-4 at the mRNA level was quantified via real time PCR. TLR-4 expression of macrophages stimulated with LPS was significantly increased following 30 minutes post stimulation (Figure 5.6A). TLR-4 expression was unaffected by TgMIF stimulation in comparison to LPS alone control (Figure 5.6B). Stimulation of macrophages with both TgMIF and LPS in combination did not effect TLR-4 expression compared with unstimulated controls (Figure 5.6C).

5.4 Discussion

In this study, the production of recombinant endotoxin-free TgMIF has allowed for the biological characterisation of this protein. Subsequently, TgMIF has been shown to be a biologically active protein capable of driving IL-8 production from human PBMC whilst having the ability to activate ERK-MAPK pathways in macrophages. By comparison, TgMIF was unable to upregulate CD74 and CD44 expression in bone marrow macrophages, except at concentrations which are not physiologically relevant. In addition, the ability of TgMIF to ablate LPS induced TLR-4 expression has been demonstrated.

MIF has been associated with many biological processes including its ability to counteract the immunosuppressive properties of GC (Calandra *et al.*, 1995) as well as being able to inhibit p53 dependent apoptosis of macrophages (Mitchell *et al.*, 2002). However, a recent study by Kudrin *et al.*, (2006) has highlighted the inconsistency in reporting of endotoxin removal procedures and endotoxin levels present in protein batches in MIF studied. For example, a study by Nguyen *et al.*, (2003) which determined the ability of MIF mutants to suppress GC activity, failed to document an LPS removal step in these experiments. Furthermore, in other work which demonstrates the ability of MIF to significantly increase the proliferation of fibroblast like synoviocytes, there is no recognition of an endotoxin removal procedure in this work (Lacey *et al.*, 2003). As a result, it is possible that studies where LPS levels in protein preparations are not documented or even indeed if a removal procedure was performed, the authenticity of these results may be called into question. Subsequently, the production of LPS-free recombinant TgMIF was essential to provide reliable and robust data. The accepted maximum level of endotoxin present in samples being used to stimulate cell cultures is a somewhat grey area as currently there is a lack of established guidelines on this matter. However, what is apparent is that accepted levels vary according to cell types being used. An initial review by Case Gould (1984) documented that 1ng/ml contaminant endotoxin was capable of activating macrophages. Later, Morris *et al.*, (1992) reported that 0.5ng/ml LPS could significantly increase IL-6 production from macrophages. Subsequently, the Profos AG endotoxin compendium has suggested that 0.01ng/ml LPS is the maximum acceptable level of endotoxin which can be present in samples being used for the stimulation of macrophages and mononuclear phagocytes. Therefore, batches of TgMIF were only ever used in these studies if LPS contamination was less than 0.01ng/ml. However, a typical batch of recombinant TgMIF contained less than 0.008ng/ml endotoxin.

Recombinant endotoxin-free TgMIF ranging from 1ng/ml to 1000ng/ml induced IL-8 production from human PBMC in a dose-dependent manner. This result is similar to that of HsMIF which has been previously shown to induce IL-8 production from THP-1 cells and human PBMC, across a concentration range between 1ng/ml and 1000ng/ml. Of note, IL-8 production peaked in PBMC following stimulation with 1ng/ml HsMIF (Kudrin *et al.,* 2006). In comparison with HsMIF, 1ng/ml TgMIF induced ~40% IL-8 from PBMC. The relativity of these results is highlighted by IL-8 function. IL-8 is secreted by many cells such as endothelial and epithelial cells in response to challenge by foreign antigen and functions mainly as a chemoattractant for cells of the immune response including macrophages but primarily neutrophils. In the acute stages of *T. gondii* infection, the rapid recruitment of neutrophils to the site of infection is essential for resolution of infection (Denkers *et al.,* 2004). IL-8 production from fibroblasts and Hela cells has been reported from cells infected with *T. gondii* tachyzoites (Denney *et al.,* 1999). Our results therefore suggest TgMIF is a strong candidate to induce this effect. The recruitment of neutrophils to the site of infection not only provides potential new host cells for the parasite, but also, neutrophils produce IL-12 which can also attract dendritic cells which have been shown in recent studies to act as Trojan horses for *T. gondii* dissemination (Lambert *et al.,* 2006, Bierly *et al.,* 2008).

Stimulation of bone marrow-derived macrophages with 100ng/ml TgMIF results in an increase in ERK MAPK phosphorylation. This result is associated with an increase in phosphorylated isoforms of p44 ERK and p42 ERK, peaking 30 minutes post stimulation. TgMIF activation of ERK-MAPK pathways is ~30 times greater than that of unstimulated controls, whereas stimulation of macrophages with 1ng/ml HsMIF increases ERK MAPK activation ~270 times. Therefore, although TgMIF is capable of significantly activating ERK MAPK, this activity is significantly reduced in comparison to that of HsMIF. Other MIF homologues have been shown to activate ERK MAPK. For example, Kamir *et al.,* (2008) demonstrated that LmMIF was capable of inducing ERK MAPK at concentrations ranging from 10ng/ml to 50ng/ml. It is also of interest that previous studies have linked activation of ERK MAPK with a subsequent increase in production of IL-8 (Buchholz and Stephens, 2007). Therefore, it would be of interest to measure ERK MAPK activation in human PBMC following stimulation with TgMIF which is associated with an increase in IL-8 production.

The MIF receptor is CD74 (Leng *et al.,* 2003), which in association with CD44 forms an active signal transduction pathway following engagement of MIF (Shi *et al.,* 2006). Stimulation of

macrophages with TgMIF resulted in a significant increase in mRNA levels of CD74 at 5µg/ml whereas no significant increase in CD44 was observed at any concentration of TgMIF tested. This is in contrast to stimulation with 1ng/ml HsMIF which results in a significant increase in both CD74 and CD44 at the mRNA level. However, to date, no separate study has been published which determines the effect of HsMIF on CD74 and CD44 expression at the mRNA and protein level in macrophages. Indeed, a study by Gore *et al.,* (2008) found an increase in CD74 mRNA expression in B cells following stimulation with 100ng/ml HsMIF for 2 hours, although, no change in CD44 mRNA levels was detected. However in these cells, CD74 and CD44 still formed a functional MIF receptor. Therefore, if these results were comparable to those observed in the macrophage, this would suggest that although TgMIF does not upregulate CD74/CD44 within physiological concentrations, TgMIF may still engage surface CD74 to form a functional MIF receptor. Furthermore, if TgMIF is capable of binding CD74, it may be a successful competitor of HsMIF for receptor binding. Subsequently, such activities may be an attempt to block anti-parasitic mechanisms which may occur as a result of HsMIF engagement.

Previous studies have documented the ability of mammalian MIF to upregulate the basal expression of TLR-4 on macrophages following stimulation (Roger *et al.,* 2001). It was therefore of interest to determine whether TgMIF was capable of inducing an effect on TLR-4 expression. TgMIF does not significantly increase or decrease TLR-4 mRNA levels following stimulation with 100ng/ml in comparison with unstimulated cells. However, intriguingly, in the presence of both TgMIF and LPS, the natural ligand for TLR-4, an apparent down regulatory effect is observed on TLR-4 mRNA levels when compared with unstimulated controls. Indeed, these results were unexpected and prompted suggestion of a potential role for TgMIF in downregulating TLR-4 expression in the presence of LPS, functioning perhaps in an anti-inflammatory capacity. These preliminary results may also imply that TgMIF may be capable of modulating macrophage responses to LPS. The ability of the parasite to control host immune responses to infection would prove beneficial to parasite survival. However, there is limited information regarding MIF regulation of TLR-4 expression (Roger *et al.,* 2001, Ohkawara *et al.,* 2005) and indeed there are no published data detailing effects of other parasite homologues of MIF on TLR-4 expression.

Chapter 6

Discussion

6.0 Discussion

Since its discovery in 1966, MIF has been described as a key pro-inflammatory cytokine involved in both innate and adaptive immune responses. Not only was MIF the first cytokine to be discovered, it is also unique in so far as it is a chemokine, a hormone and it has enzyme activities which may mediate biological functions. Furthermore, a role for MIF as a potent mediator in human diseases including rheumatoid arthritis and cancers has also been described.

The MIF gene is found in a great variety of different species including *M. musculus*, *Rattus norvegicus*, *Arabidopsis thaliana*, *Danio rerio* and *Xenopus laevis*. Homologues of MIF have also been identified in several parasitic species including *L. major*, *T. spiralis* and *A. ceylanicum* and indeed the first published study of a parasitic MIF homologue detailed the characterisation of *B. malayi* MIF (Pastrana *et al.,* 1998).

The research presented in this thesis describes the first account of a homologue of MIF found in the *T. gondii* genome. Bioinformatical analysis of TgMIF found in the genomes of four different strains of *T. gondii*, revealed great sequence conservation between strains with a single nucleotide substitution being the only highlighted difference. Sequence conservation can be an indication of the importance of a particular gene and indeed the fact that TgMIF has not been removed from the genome due to evolutionary selection pressures or even undergone any substantial mutational events, suggests that TgMIF performs an important role with regard to the ability of a the parasite to initiate a successful infection.

Further bioinformatical analysis also revealed that MIF is conserved within other species although MIF homologues have not been identified in all Apicomplexa including *C. parvum*, *T. parvum* and *T. annulata.* Despite this, MIF sequences have been identified in other Apicomplexa such as *P. falciparum* and *N. caninum* and indeed, TgMIF shares greatest homology with these species. However greatest sequence conservation is observed between *T. gondii* and the closely related species *N. caninum* whereby over 76% conservation is observed.

Preliminary experiments suggest that at the mRNA level, TgMIF is expressed in both tachyzoites and bradyzoites. Furthermore, although unquantifiable, TgMIF expression at the mRNA level appears to be greater in tachyzoites than bradyzoites. However, further studies are required to determine whether this translates through to the protein level. MIF expression has also been shown to vary at different stages presented throughout the life cycle of other

Apicomplexa (Augustijn *et al.*, 2007, Miska *et al.*, 2007). It would therefore be of interest to further study the stage-specific expression of TgMIF throughout the *T. gondii* life cycle, which may provide an indication of protein function.

Following generation of anti-TgMIF antibodies, it was possible to identify TgMIF as a protein found in the secretory products of tachyzoites cultured *in vitro*. To date, the mechanism by which MIF/TgMIF is secreted is unclear as these proteins lack N-terminal signal sequences which are required for conventional protein secretion. With recent literature suggesting a role for the golgi protein p115 in human MIF secretion, it was anticipated that there may be a p115 homologue in the *T. gondii* genome. However, a search of the *T. gondii* genome using a variety of p115 sequences from mammals, helminths, bacteria e.t.c. did not produce any significant hits. Further searches also suggested that p115 homologues were not present in the genomes of other protozoan parasites such as *Plasmodium* and *Leishmania*. Subsequently, it may be possible that secretion of MIF homologues in these protozoan parasites is independent of p115.

Cloning, expression and purification of TgMIF allowed for the enzymatic characterisation of TgMIF to be carried out under optimised conditions. TgMIF tautomerase activity was measured by the conversion of L.DOPA methyl ester to DHICA which was 91 fold lower than the measured activity for HsMIF. This significantly reduced tautomerase activity is not unlike what has been measured for other homologues (Kamir *et al.*, 2008, Richardson *et al.*, 2009, Cho *et al.*, 2007). Previous reports have speculated that MIF biological function may be mediated via its enzyme activities (Swope *et al.*, 1998, Lubetsky *et al.*, 2002). As TgMIF is a potent stimulator of IL-8 production but has relatively weak enzymatic activity, it could indicate that this biological activity is independent of enzyme activities closely associated with MIF. Conversely, conservation of the active site but not necessarily the enzyme function, may be all that is required for immunological activity. Future studies will elucidate the mode of action of TgMIF and determine whether enzyme activities and IL-8 production are linked.

Characterisation of TgMIF tautomerase activity has involved the thorough optimisation of assay conditions including parameters such as the selection of an appropriate buffer and pH. This study has also highlighted the need to carry out storage assays as TgMIF tautomerase activity quickly deteriorates following purification. Furthermore, the lack of fully comprehensive enzyme studies in MIF homologues, with the exception of *T. spiralis* (Tan *et*

al., 2001), makes it difficult to compare the efficiency of TgMIF as an enzyme in comparison with other homologues.

MIF oxidoreductase activity is less well researched and indeed, in the case of parasite homologues, this activity is often not discussed (Cho *et al.,* 2007, Zang *et al.,* 2002, Kamir *et al.,* 2008). This is perhaps due to evidence which suggests that both conserved cysteines are required for optimal oxidoreductase activity (Kleemann *et al.,* 1998) and it is often the case that in many homologues, one of these cysteines is absent (Cho *et al.,* 2007, Miska *et al.,* 2007, Kamir *et al.,* 2008). Characterisation of TgMIF oxidoreductase activity shows that this activity is virtually undetectable, even under optimised assay conditions. This is similar to LmMIF which has a small yet detectable activity (Richardson *et al.,* 2009).

The production of recombinant TgMIF also allowed for the crystal structure of this protein to be determined. TgMIF structure was determined by crystallography (see appendix F). As expected, the global topology of TgMIF is similar to that of HsMIF in that the protein forms a trimeric architecture, whereby each monomer consists of a four stranded beta sheet with two alpha helices. However, there are subtle differences in the TgMIF structure compared to HsMIF, most notably substitutions in and around the active site. These substitutions result in steric hindrance of the tautomerase active site which may account for the reduction in TgMIF affinity for L.DOPA methyl ester in comparison to HsMIF as discussed in chapter 3. Furthermore, these differences also prevent successful binding of MIF inhibitor ISO-1 and consequently TgMIF tautomerase activity is unaffected following incubation with ISO-1.

As discussed earlier, some previous studies which aimed to characterise aspects of MIF function, failed to document an endotoxin removal procedure and consequently the LPS levels present in experimental samples. Consequently, the results obtained may be due to endotoxin contamination and not necessarily be a direct effect of MIF. However, based on the studies discussed in chapter 5, the work carried out in this thesis was performed using endotoxin-free recombinant TgMIF which had minimum levels of LPS less than 0.01ng/ml, thus providing reliable results.

Endotoxin-free recombinant TgMIF has the ability to induce IL-8 production from human PBMC. Furthermore, an increase in ERK MAPK activation following TgMIF stimulation complements the increase of IL-8 produced in stimulated cells. Despite weaker ERK MAPK

activation being measured following TgMIF stimulation in comparison to HsMIF, this may also account for HsMIF being a more efficient mediator of IL-8 production. In addition, this pattern of IL-8 induction has been observed in studies with other MIF homologues. Both *B. malayi* MIF homologues have been shown to induce IL-8 production from human monocytes, with significant results being obtained following stimulation with 1μM BmMIF (Zang *et al.,* 2002). Furthermore, this study also highlighted the ability of BmMIFs to induce production of endogenous HsMIF from monocytes. This observation prompted suggestion that a positive feedback loop may be in place. In addition, previous evidence has indicated that at high concentrations of MIF, AP-1 dependent pro-inflammatory gene expression is blocked (Kleemann *et al.,* 2000). Therefore, it would be of interest to further analyse cell supernatants from human PBMC stimulated with TgMIF to assess HsMIF levels. An increase in HsMIF production at the site of infection in synergy with parasite secreted TgMIF, may facilitate a local environment in which pro-inflammatory gene expression is inhibited due to high concentrations of MIF. This situation may counter regulate others which have been previously discussed regarding TgMIF induced IL-8 production whereby the pro-inflammatory environment created by IL-8 is balanced by MIF induced AP-1 inhibition, ultimately creating a regulated environment which is optimal for parasite survival.

The MIF receptor is CD74 which in association with CD44 can facilitate signal transduction (Leng *et al.,* 2003, Shi *et al.,* 2006). In this study, TgMIF did not significantly upregulate either CD74 or CD44 expression on macrophages at physiological concentrations. However, CD74 and CD44 are common cell surface glycoproteins and subsequently upregulation of these proteins may be unnecessary (Goodison *et al.,* 1999). These results however, do not rule out the possibility of TgMIF being a suitable binding partner for CD74, and indeed it would be of interest to assess the interaction between TgMIF and CD74. Studies in other parasite homologues such as *L. major* and *A. ceylanicum* have shown that by measuring real time binding interactions, both LmMIF and AcMIF bind CD74 (Kamir *et al.,* 2008, Cho *et al.,* 2007). In particular, LmMIF and AcMIF bind CD74 with 3 and 4.5 fold lower affinity than HsMIF respectively (Kamir *et al.,* 2008, Cho *et al.,* 2007).

Mammalian MIF upregulates TLR-4 expression on stimulated macrophages (Roger *et al.,* 2001). Stimulation of murine macrophages with TgMIF did not increase TLR-4 expression, although, macrophages stimulated with both TgMIF and LPS had mRNA TLR-4 levels similar to those unstimulated controls. This suggests a potential anti-inflammatory function for

TgMIF in down regulating TLR-4 expression on LPS stimulated macrophages. Indeed, of interest, *T. gondii* has ligands such as HSP70 (Aosai *et al.*, 2006) which are capable of binding TLR-4. However, further experimentation is required to confirm this hypothesis. Of note, it would be of interest to observe the effects of TLR-4 expression on macrophages stimulated with decreasing concentrations of TgMIF with LPS.

Within this thesis the immunomodulatory properties of TgMIF are well documented and subsequently a role for TgMIF in *T. gondii* infection should not be dismissed. As discussed in chapter 1, there are several described immunomodulatory mechanisms and molecules which are released by *T. gondii* during infection, and although not described elsewhere, it may be possible that TgMIF may act with other immunomodulatory molecules secreted by *T. gondii* during infection in order to augment a particular response which best favours parasite survival.

To conclude, in this thesis the first description of the *T. gondii* homologue of human MIF is described. The crystal structure of TgMIF has been solved and subsequently, TgMIF shares the same global topology as HsMIF whereby it exists as a trimer composed of three identical 12.5KDa monomers. Furthermore, TgMIF has been successfully biochemically characterised in a study which demonstrated a functional tautomerase activity but an inactive oxidoreductase activity. In addition, endotoxin free, enzymatically active TgMIF has been shown to be an immunologically active protein which is capable of inducing IL-8 production from human PBMC whilst inducing ERK MAPK activation in murine macrophages. Although recombinant TgMIF did not effect CD74 or CD44 expression on bone marrow derived macrophages, TLR-4 expression appeared to be downregulated following simultaneous stimulation of macrophages with TgMIF and LPS.

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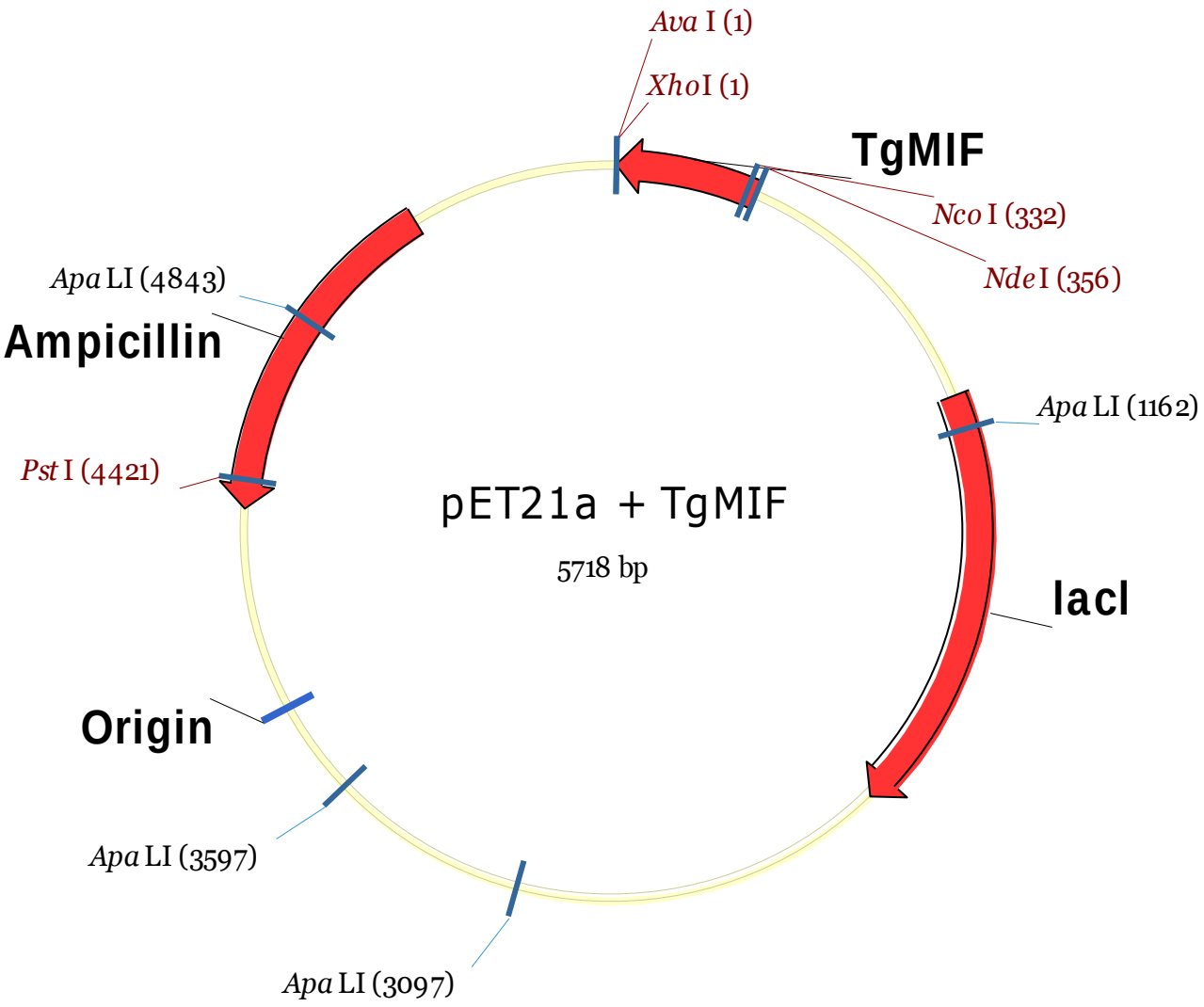
Appendices

Gene	Forward Primer Sequence	Reverse Primer Sequence	Product Size (nt)	Annealing Temperature (°C)
TgMIF	GGG GCA TAT GCC CAA GTG CAT GAT CTT TTG CC	GGG GCT CGA GGC CGA AAG TTC GGT CGC CCA TGG C	361	64
TgMIF (Internal)	TCT TGA AGG ACG CCG AAA AAG	AAC TGG TGA TGC CTC CAA TGG	164	64
TBP	AAC AGC AGC AGC AAC AAC AGC AGG	TGA TAG GGG TCA TAG GAG TCA TTG G	192	64
CD74	GCT TCC GAA ATC TGC CAA ACC	AAG AGC CAC TGC TTC ATC CAG C	279	64
CD44	CCT GGA ATA AAA ACC ACA GCC AAC	TAG TCG CCT CTG GAA AAG GGT C	188	64
TLR-4	AGC AAA GTC CCT GAT G ACA TTC C	CCA CAG CCA CCA GAT TCT CTA AAC	268	64
pet21a Sequencing Primers	TAA TAC GAC TCA CTA TAGGG	GCT AGT TAT TGC TCA GCGG	-	-

Appendix A

Table A: Oligonucleotide primers were designed using MacVector 7.0 (Oxford Biomolecular, UK) and synthesised by Invitrogen (Paisley, UK).



Appendix B

Figure B: Vector map of pET21a containing TgMIF between restriction enzyme sites *Xho*I and *Nde*I.

```
                        1                                                  50
G. lamblia p115   (1) ----MGLVSAQNLQPVQLDASEKIVAIHPTNTDVMVLTQKPDPGSQARDY
Hsp115            (1) MNFLRGVMGGQSAGPQHTEA-ETIQKLCDRVASSTLLDDRRNAVRALKSL
Mmp115            (1) MNFLRGVMGGQSAGPQHTEA-ETIQKLCDRVASSTLLDDRRNAVRALKSL
Consensus         (1) MNFLRGVMGGQSAGPQHTEA ETIQKLCDRVASSTLLDDRRNAVRALKSL

                       51                                                 100
G. lamblia p115  (47) LSSSYLLYSLVNQEPVFSFKGLLLFVASDMSRIVRFSGHAIYDYEQEPAD
Hsp115           (50) SKKYRLEVGIQAMEHLIHVLQTDRSDSEIIGYALDILYNIISNEEEEEVE
Mmp115           (50) SKKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALDTLYNIISNDEEEELE
Consensus        (51)  SKKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALDLYNIISNDEEEELE

                      101                                                 150
G. lamblia p115  (97) TSGRVAYRVKNKIRVGTFIPAIGAIAKSEKDGREYFVYAGLTTRRQADTL
Hsp115          (100) ENSTRQSEDLGSQFTEIFIKQQENVTLLLSLLEEFDFHVRWPGVKLLTSL
Mmp115          (100) ENSTRQSEDLGSQFTEIFIKQPENVTLLLSLLEEFDFHVRWPGVRLLTSL
Consensus       (101) ENSTRQSEDLGSQFTEIFIKQ ENVTLLLSLLEEFDFHVRWPGVRLLTSL

                      151                                                 200
G. lamblia p115 (147) SYFHQNDHSRPEKVMHMYYGYAIIDLQSPKAAESLVRTCILPIKRRNQ--
Hsp115          (150) LKQLGPQVQQIILVSPMGVSRLMDLLADSREVIRNDGVLLLQALTRSNGA
Mmp115          (150) LKQLGPPVQQIILVSPMGVSRLMDLLADSREIIRNDGVLLLQALTRSNGA
Consensus       (151) LKQLGP VQQIILVSPMGVSRLMDLLADSREIIRNDGVLLLQALTRSNGA

                      201                                                 250
G. lamblia p115 (195) IFSIRPNVDANKLFISYINSKS------------LMLEIALDLDKGYKMY
Hsp115          (200) IQKIVAFENAFERLLDIISEEGNSDGGIVVEDCLILLQNLLKNNNSNQNF
Mmp115          (200) IQKIVAFENAFERLLDIITEEGNSDGGIVVEDCLILLQNLLKTNNSNQNF
Consensus       (201) IQKIVAFENAFERLLDIISEEGNSDGGIVVEDCLILLQNLLK NNSNQNF

                      251                                                 300
G. lamblia p115 (233) FKDEDTPIQYAPSDDSGHFIYPYLLQPETAKHTMHQNILHMSSDIT---F
Hsp115          (250) FKEGSYIQRMKPWFEVGDENSGWSAQKVTNLHLMLQLVRVLVSPTNPPGA
Mmp115          (250) FKEGSYIQRMKPWFEVGEENSGWSAQKVTNLHLMLQLVRVLVSPTNPPGA
Consensus       (251) FKEGSYIQRMKPWFEVGDENSGWSAQKVTNLHLMLQLVRVLVSPTNPPGA

                      301                                                 350
G. lamblia p115 (280) IRISRDSLFLDIIYNQVTRETLWHLFAVDLAASASSTTQATES--MTTED
Hsp115          (300) TSSCQKAMFQCGLLQQLCTILMATGVPADILTETINTVSEVIRGCQVNQD
Mmp115          (300) TSSCQKAMFQCGLLQQLCTILMATGIPADILTETINTVSEVIRGCQVNQD
Consensus       (301) TSSCQKAMFQCGLLQQLCTILMATGIPADILTETINTVSEVIRGCQVNQD

                      351                                                 400
G. lamblia p115 (328) LNASSNTVTYG-TYAFEIVRNGGVFPFLPSGSLYYHDYSTNTLQYISKKK
Hsp115          (350) YFASVNAPSNPPRPAIVVLLMSMVNERQPFVLRCAVLYCFQCFLYKNQKG
Mmp115          (350) YFASVNAPSNPPRPAIVVLLMSMVNERQPFVLRCAVLYCFQCFLYKNEKG
Consensus       (351) YFASVNAPSNPPRPAIVVLLMSMVNERQPFVLRCAVLYCFQCFLYKN KG

                      401                                                 450
G. lamblia p115 (377) IEKFMHSDMIGKCACKWNTEEIAFFINTQELAMGTDRVVQLTDDLFKLYK
Hsp115          (400) QGEIVSTLLPSTIDATGNSVSAGQLLCGGLFSTDSLSNWCAAVALAHALQ
Mmp115          (400) QGEIVATLLPSTIDATGNSVSAGQLLCGGLFSTDSLSNWCAAVALAHALQ
Consensus       (401) QGEIVATLLPSTIDATGNSVSAGQLLCGGLFSTDSLSNWCAAVALAHALQ

                      451                                                 500
G. lamblia p115 (427) HTARKLVPAQDALQGSMTGFNINTTYAPNVLAMSAPQPSHELLREL GEMK
Hsp115          (450) ENATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQGSKIQTRVGLLMLLC
Mmp115          (450) GNATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQGSKIQTRVGLLMLLC
Consensus       (451)  NATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQGSKIQTRVGLLMLLC
```

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                          501                                                 550
G. lamblia p115  (477)TATKNTDEALETIKKELETLKEVKARQGDDLDSLK------TAMEANDAN
Hsp115           (500)TWLSNCPIAVTHFLHNSANVPFLTGQIAENLGEEEQLVQGLCALLLGISI
Mmp115           (500)TWLSNCPIAVTHFLHNSANVPFLTGQIAENLGEEEQLVQGLCALLLGISI
Consensus        (501)TWLSNCPIAVTHFLHNSANVPFLTGQIAENLGEEEQLVQGLCALLLGISI

                          551                                                 600
G. lamblia p115  (521)YKNIYDAVEELKERQEELQAAESLASGTLDPELLEKIHAAADMAQQADLI
Hsp115           (550)YFNDNSLESYMKEKLKQLIEKRIGKENFIEKLGFISKHELYSRASQKPQP
Mmp115           (550)YFNDNSLENYTKEKLKQLIEKRIGKENFIEKLGFISKHELYSRASQKPQP
Consensus        (551)YFNDNSLE YLKEKLKQLIEKRIGKENFIEKLGFISKHELYSRASQKPQP

                          601                                                 650
G. lamblia p115  (571)RNQVLEMDGRMAEVETQAAKLAAEVQELEKNYGE-ERASAAMLGDSEAIM
Hsp115           (600)NFPSPEYMIFDHEFTKLVKELEGVITKAIYKSSEEDKKEEEVKKTLEQHD
Mmp115           (600)NFPSPEYMIFDHEFTKLVKELEGVITKAIYKSSEEDKKEEEVKKTLEQHD
Consensus        (601)NFPSPEYMIFDHEFTKLVKELEGVITKAIYKSSEEDKKEEEVKKTLEQHD


                          651                                                 700
G. lamblia p115  (620)AIVSEVKGDVSALKDQVRDQGEHIAAIQDDYNGYKEAVNNRFVILEETVL
Hsp115           (650)NIVTHYKNMIREQDLQLEELRQQVSTLKCQNEQLQTAVTQQVSQIQQHKD
Mmp115           (650)NIVTHYKNMIREQDLQLEELKQQVSTLKCQNEQLQTAVTQQASQIQQHKD
Consensus        (651)NIVTHYKNMIREQDLQLEELKQQVSTLKCQNEQLQTAVTQQ SQIQQHKD

                          701                                                 750
G. lamblia p115  (670)EIQRNIPIEDEDSFDFDPHSIRIDEEGADDEAQENRVRNKLLTDNDDDDD
Hsp115           (700)QYNLLKIQLGKDNQHQGSYSEGAQMNGIQPEE-IGRLREEIEELKRNQEL
Mmp115           (700)QYNLLKVQLGKDNHHQGSHGDGAQVNGIQPEE-ISRLREEIEELKSQQAL
Consensus        (701)QYNLLKIQLGKDN HQGSHSDGAQMNGIQPEE I RLREEIEELK NQDL

                          751                                                 800
G. lamblia p115  (720)LVGTPNVQQMRVVPK---ERVEQIPPVSEKLPVQDAEPIEPLQMHERVQE
Hsp115           (749)LQSQLTEKDSMIENMKSSQTSGTNEQSSAIVSARDSEQVAELKQELATLK
Mmp115           (749)LQGQLAEKDSLIENLKSSQASGMSEQASATCPPRDPEQVAELKQELTALK
Consensus        (751)LQGQL EKDSLIENLKSSQ SG  EQASA LP RDAEQVAELKQEL  LK

                          801                                                 850
G. lamblia p115  (767)IQS-PPKLVKTEVEDPVEHWGPDLVERLPDVPPSDDGDEPPISSVTEAAP
Hsp115           (799)SQLNSQSVEITKLQTEKQELLQKTEAFAKSVEVQGETETIIATKTTDVEG
Mmp115           (799)SQLCSQSLEITRLQTENCELLQRAETLAKSVPVEGESEHVSAAKTTDVEG
Consensus        (801)SQL SQSLEITKLQTE  ELLQK E  AKSVPV GESE I ASKTTDVEG

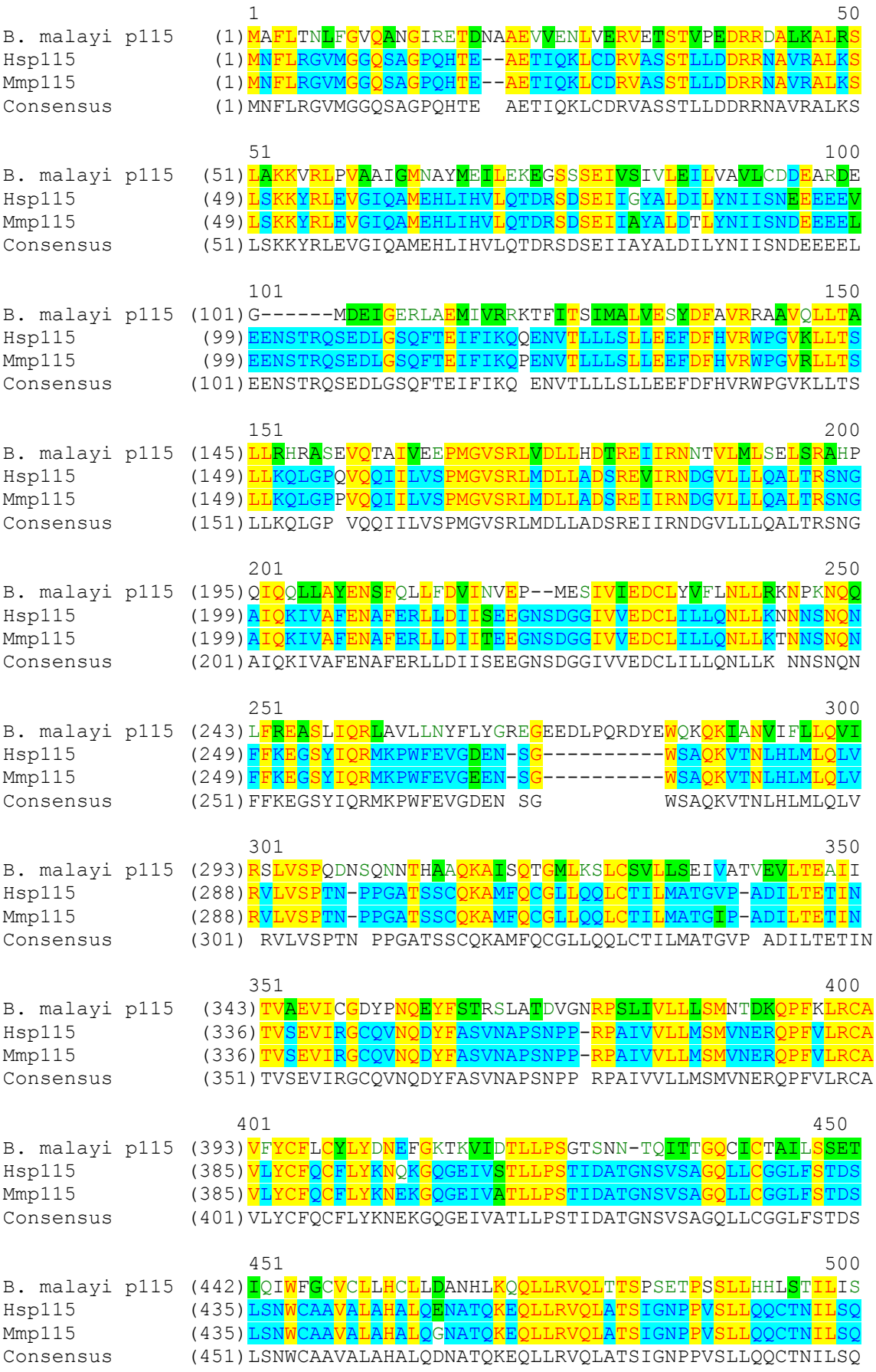
                          851                                                 900
G. lamblia p115  (816)VATTTLDAFSDLDLVGSPVETKQGSAPATTEPQNSNTMQSPQIDFEDLSV
Hsp115           (849)RLSALLQETKELKNEIKALSEERTAIKEQLDSSNS-TIAILQTEKDKLEL
Mmp115           (849)RLSALLQETKELKNEIKALSEERTAIQKQLDSSNS-TIAILQTEKDKLDL
Consensus        (851)RLSALLQETKELKNEIKALSEERTAI  QLDSSNS TIAILQTEKDKLDL


                          901                                                 950
G. lamblia p115  (866)GGWTGSPDPKPCLETLPNPLQSPGDEFEKLTKDDFDLGESATLNPNAEGD
Hsp115           (898)EITDSKKEQDDLLVLLADQDQKILSLKNKLKDLGHPVEEEDELESGDQED
Mmp115           (898)EVTDSKKEQDDLLVLLADQDQKILSLKSKLKDLGHPVEEED-ESGDQE-D
Consensus        (901) EITDSKKEQDDLLVLLADQDQKILSLK KLKDLGHPVEEEDLESGDQED

                          951          967
G. lamblia p115  (916)AGQDVDDFAGDFDDFDI
Hsp115           (948)EDDESEDPGKDLDHI--
Mmp115           (946)DDDEIDDGDKDQDI---
Consensus        (951)DDDEIDD AKD D
```

Appendix C

Figure C1: Alignment of human (CAA55394) and murine p115 (AAH16069) with *G. lamblia* (ED080210) p115 in which 10.8% identity is conserved. Identical amino acids are highlighted in yellow blocks, conservative residues are highlighted in aqua blocks, blocks of similar amino acids are highlighted in green blocks, non-identical amino acids are highlighted in white blocks.


1 50
B. malayi p115 (1)MAFLTNLFGVQANGIRETDNAAEVVENLVERVETSTVPEDRRDALKALRS
Hsp115 (1)MNFLRGVMGGQSAGPQHTE--AETIQKLCDRVASSTLLDDRRNAVRALKS
Mmp115 (1)MNFLRGVMGGQSAGPQHTE--AETIQKLCDRVASSTLLDDRRNAVRALKS
Consensus (1)MNFLRGVMGGQSAGPQHTE AETIQKLCDRVASSTLLDDRRNAVRALKS
51 100
B. malayi p115 (51)LAKKVRLPVAAIGMNAYMEILEKEGSSSEIVSIVLEILVAVLCDDEARDE
Hsp115 (49)LSKKYRLEVGIQAMEHLIHVLQTDRSDSEIIGYALDILYNIISNEEEEEV
Mmp115 (49)LSKKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALDTLYNIISNDEEEEL
Consensus (51)LSKKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALDILYNIISNDEEEEL
101 150
B. malayi p115 (101)G------MDEIGERLAEMIVRRKTFITSIMALVESYDFAVRRAAVQLLTA
Hsp115 (99)EENSTRQSEDLGSQFTEIFIKQQENVTLLLSLLEEFDFHVRWPGVKLLTS
Mmp115 (99)EENSTRQSEDLGSQFTEIFIKQPENVTLLLSLLEEFDFHVRWPGVRLLTS
Consensus (101)EENSTRQSEDLGSQFTEIFIKQ ENVTLLLSLLEEFDFHVRWPGVKLLTS
151 200
B. malayi p115 (145)LLRHRASEVQTAIVEEPMGVSRLVDLLHDTREIIRNNTVLMLSELSRAHP
Hsp115 (149)LLKQLGPQVQQIILVSPMGVSRLMDLLADSREVIRNDGVLLLQALTRSNG
Mmp115 (149)LLKQLGPPVQQIILVSPMGVSRLMDLLADSREIIRNDGVLLLQALTRSNG
Consensus (151)LLKQLGP VQQIILVSPMGVSRLMDLLADSREIIRNDGVLLLQALTRSNG
201 250
B. malayi p115 (195)QIQQLLAYENSFQLLFDVINVEP--MESIVIEDCLYVFLNLLRKNPKNQQ
Hsp115 (199)AIQKIVAFENAFERLLDIISEEGNSDGGIVVEDCLILLQNLLKNNNSNQN
Mmp115 (199)AIQKIVAFENAFERLLDIITEEGNSDGGIVVEDCLILLQNLLKTNNSNQN
Consensus (201)AIQKIVAFENAFERLLDIISEEGNSDGGIVVEDCLILLQNLLK NNSNQN
251 300
B. malayi p115 (243)LFREASLIQRLAVLLNYFLYGREGEEDLPQRDYEWQKQKIANVIFLLQVI
Hsp115 (249)FFKEGSYIQRMKPWFEVGDEN-SG----------WSAQKVTNLHLMLQLV
Mmp115 (249)FFKEGSYIQRMKPWFEVGEEN-SG----------WSAQKVTNLHLMLQLV
Consensus (251)FFKEGSYIQRMKPWFEVGDEN SG WSAQKVTNLHLMLQLV
301 350
B. malayi p115 (293)RSLVSPQDNSQNNTHAAQKAISQTGMLKSLCSVLLSEIVATVEVLTEAII
Hsp115 (288)RVLVSPTN-PPGATSSCQKAMFQCGLLQQLCTILMATGVP-ADILTETIN
Mmp115 (288)RVLVSPTN-PPGATSSCQKAMFQCGLLQQLCTILMATGIP-ADILTETIN
Consensus (301) RVLVSPTN PPGATSSCQKAMFQCGLLQQLCTILMATGVP ADILTETIN
351 400
B. malayi p115 (343)TVAEVICGDYPNQEYFSTRSLATDVGNRPSLIVLLLSMNTDKQPFKLRCA
Hsp115 (336)TVSEVIRGCQVNQDYFASVNAPSNPP-RPAIVVLLMSMVNERQPFVLRCA
Mmp115 (336)TVSEVIRGCQVNQDYFASVNAPSNPP-RPAIVVLLMSMVNERQPFVLRCA
Consensus (351)TVSEVIRGCQVNQDYFASVNAPSNPP RPAIVVLLMSMVNERQPFVLRCA
401 450
B. malayi p115 (393)VFYCFLCYLYDNEFGKTKVIDTLLPSGTSNN-TQITTGQCICTAILSSET
Hsp115 (385)VLYCFQCFLYKNQKGQGEIVSTLLPSTIDATGNSVSAGQLLCGGLFSTDS
Mmp115 (385)VLYCFQCFLYKNEKGQGEIVATLLPSTIDATGNSVSAGQLLCGGLFSTDS
Consensus (401)VLYCFQCFLYKNEKGQGEIVATLLPSTIDATGNSVSAGQLLCGGLFSTDS
451 500
B. malayi p115 (442)IQIWFGCVCLLHCLLDANHLKQQLLRVQLTTSPSETPSSLLHHLSTILIS
Hsp115 (435)LSNWCAAVALAHALQENATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQ
Mmp115 (435)LSNWCAAVALAHALQGNATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQ
Consensus (451)LSNWCAAVALAHALQDNATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQ

```
                      501                                                 550
B. malayi p115 (492)LGNRRPQTRTGLLMLLSTWLHNCPLAVAQFISIEENVQYLTTHIDGYGNE
Hsp115         (485)GS--KIQTRVGLLMLLCTWLSNCPIAVTHFLHNSANVPFLTGQIAENLGE
Mmp115         (485)GS--KIQTRVGLLMLLCTWLSNCPIAVTHFLHNSANVPFLTGQIAENLGE
Consensus      (501)GS  KIQTRVGLLMLLCTWLSNCPIAVTHFLHNSANVPFLTGQIAENLGE

                      551                                                 600
B. malayi p115 (542)GSEAENQIVRGLIAFLLTVCLVFDETGVDQSRKNALSVVVERRVGKEKLV
Hsp115         (533)----EEQLVQGLCALLLGISIYFNDNSLESYMKEKLKQLIEKRIGKENFI
Mmp115         (533)----EEQLVQGLCALLLGISIYFNDNSLENYTKEKLKQLIEKRIGKENFI
Consensus      (551)    EEQLVQGLCALLLGISIYFNDNSLENY KEKLKQLIEKRIGKENFI

                      601                                                 650
B. malayi p115 (592)ELLEGFSHSEHYVKAAQRPQPLAKSAQDLLLDYHFTKFFKSVEGQMVKQL
Hsp115         (579)EKLGFISKHELYSRASQKPQPNFPSPEYMIFDHEFTKLVKELEGVITKAI
Mmp115         (579)EKLGFISKHELYSRASQKPQPNFPSPEYMIFDHEFTKLVKELEGVITKAI
Consensus      (601)EKLGFISKHELYSRASQKPQPNFPSPEYMIFDHEFTKLVKELEGVITKAI

                      651                                                 700
B. malayi p115 (642)CPSADISSSSS-------NNDSVFNSYKELIKRQDETIASLNQQIRKLKE
Hsp115         (629)YKSSEEDKKEEEVKKTLEQHDNIVTHYKNMIREQDLQLEELRQQVSTLKC
Mmp115         (629)YKSSEEDKKEEEVKKTLEQHDNIVTHYKNMIREQDLQLEELKQQVSTLKC

Consensus      (651)YKSSEEDKKEEEVKKTLEQHDNIVTHYKNMIREQDLQLEELKQQVSTLKC

                      701                                                 750
B. malayi p115 (685)EAE-----------NQQ-----------NYDKENELASLWRQLAEQCQVN
Hsp115         (679)QNEQLQTAVTQQVSQIQQHKDQYNLLKIQLGKDNQHQGSYSEGAQMNGIQ
Mmp115         (679)QNEQLQTAVTQQASQIQQHKDQYNLLKVQLGKDNHHQGSHGDGAQVNGIQ
Consensus      (701)QNEQLQTAVTQQ SQIQQHKDQYNLLKIQLGKDN HQGSW DGAQMNGIQ

                      751                                                 800
B. malayi p115 (713)ETSISRQ-QEIEHYKCMTVQWQSEAKRYQKWAEQWQQYQVAQSP------
Hsp115         (729)PEEIGRLREEIEELKRNQELLQSQLTEKDSMIENMKSSQTSGTNEQSSAI
Mmp115         (729)PEEISRLREEIEELKSQQALLQGQLAEKDSLIENLKSSQASGMSEQASAT
Consensus      (751)PEEISRLREEIEELK NQ LLQSQL EKDSLIENLKSSQ SGS EQASA

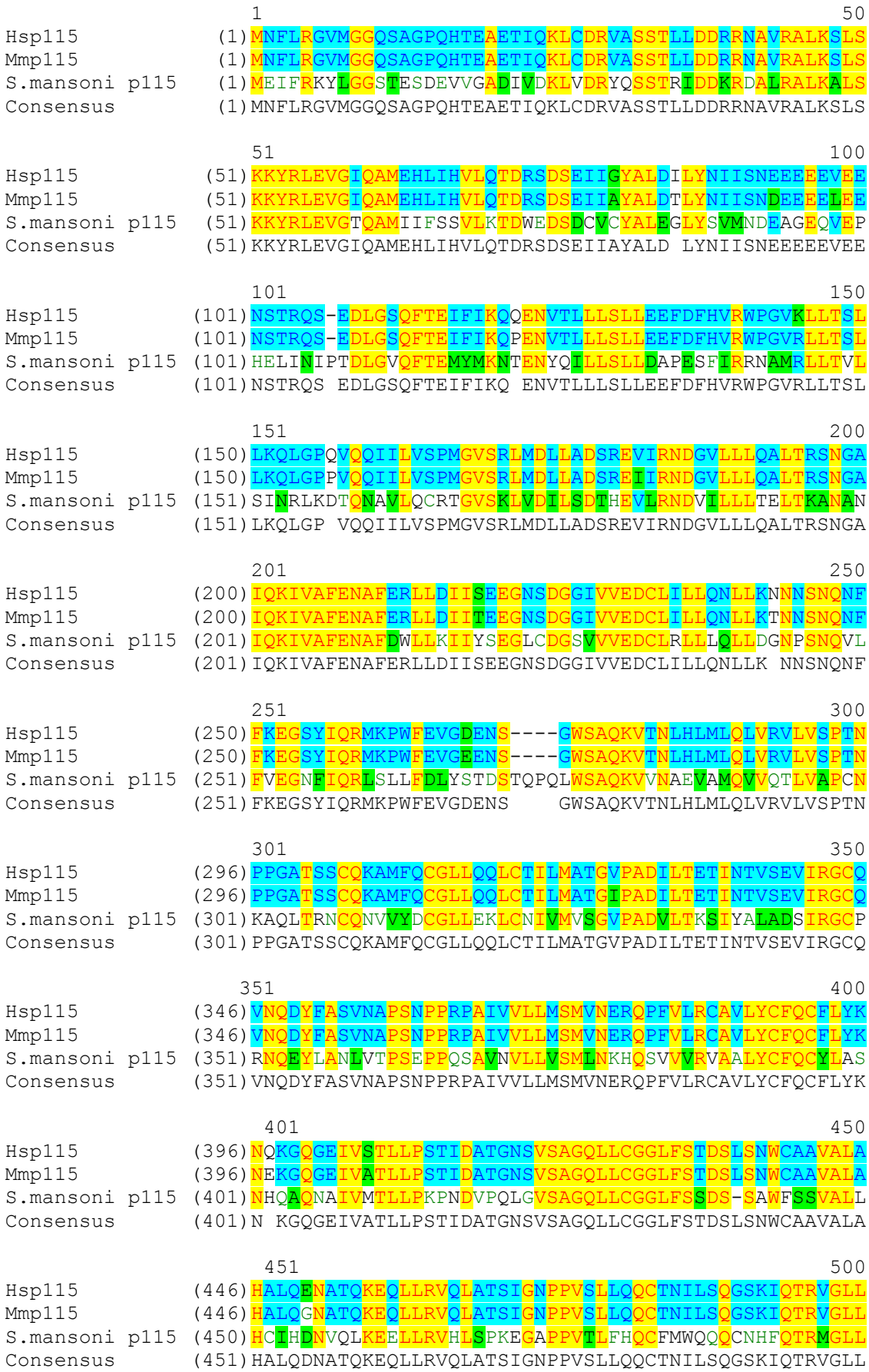
                      801                                                 850
B. malayi p115 (756)-------------------NP-----QDPLVMQLISQNKELEEQLERGWQM
Hsp115         (779)VSARDSEQVAELKQELATLKSQLNSQSVEITKLQTEKQELLQKTEAFAKS
Mmp115         (779)CPPRDPEQVAELKQELTALKSQLCSQSLEITRLQTENCELLQRAETLAKS
Consensus      (801)   RD EQVAELKQEL  LKSQL SQSLEITKLQTEN ELLQK E  AKS

                      851                                                 900
B. malayi p115 (783)YDSQG-----VS---------LVAALTEIEEAKTKIRVLEG--QIAQTVL
Hsp115         (829)VEVQGETETIIATKTTDVEGRLSALLQETKELKNEIKALSEERTAIKEQL
Mmp115         (829)VPVEGESEHVSAAKTTDVEGRLSALLQETKELKNEIKALSEERTAIQKQL
Consensus      (851)VDVQGESE IIA KTTDVEGRLSALLQETKELKNEIKALSEERTAIQ QL

                      901                                                 950
B. malayi p115 (817)DHS---YKQMNSTEIVNDDEVAVLRKEQEDLLVLLADQDAKILEYRRKLT
Hsp115         (879)DSSNSTIAILQTEKDKLELEITDSKKEQDDLLVLLADQDQKILSLKNKLK
Mmp115         (879)DSSNSTIAILQTEKDKLDLEVTDSKKEQDDLLVLLADQDQKILSLKSKLK
Consensus      (901)DSSNSTIAILQTEKDKLDLEVTDSKKEQDDLLVLLADQDQKILSLK KLK
                        951                             984
B. malayi p115 (864)ELGQAVTDDEDTDA--------------------
Hsp115         (929)DLGHPVEEEDELESGDQEDEDDESEDPGKDLDHI
Mmp115         (929)DLGHPVEEED--ESGDQEDDDDEIDDGDKDQDI-
Consensus      (951)DLGHPVEEEDD ESGDQEDDDDE DD  KD D
```

Appendix C

Figure C2: Alignment of human (CAA55394) and murine p115 (AAH16069) with *B. malayi* (XP_001902351) p115 in which 32.1% identity is conserved. Identical amino acids are highlighted in yellow blocks, conservative residues are highlighted in aqua blocks, blocks of similar amino acids are highlighted in green blocks, non-identical amino acids are highlighted in white blocks.


1 50
Hsp115 (1)MNFLRGVMGGQSAGPQHTEAETIQKLCDRVASSTLLDDRRNAVRALKSLS
Mmp115 (1)MNFLRGVMGGQSAGPQHTEAETIQKLCDRVASSTLLDDRRNAVRALKSLS
S.mansoni p115 (1)MEIFRKYLGGSTESDEVVGADIVDKLVDRYQSSTRIDDKRDALRALKALS
Consensus (1)MNFLRGVMGGQSAGPQHTEAETIQKLCDRVASSTLLDDRRNAVRALKSLS
51 100
Hsp115 (51)KKYRLEVGIQAMEHLIHVLQTDRSDSEIIGYALDILYNIISNEEEEEVEE
Mmp115 (51)KKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALDTLYNIISNDEEEELEE
S.mansoni p115 (51)KKYRLEVGTQAMIIFSSVLKTDWEDSDCVCYALEGLYSVMNDEAGEQVEP
Consensus (51)KKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALD LYNIISNEEEEEVEE
101 150
Hsp115 (101)NSTRQS-EDLGSQFTEIFIKQQENVTLLLSLLEEFDFHVRWPGVKLLTSL
Mmp115 (101)NSTRQS-EDLGSQFTEIFIKQPENVTLLLSLLEEFDFHVRWPGVRLLTSL
S.mansoni p115 (101)HELINIPTDLGVQFTEMYMKNTENYQILLSLLDAPESFIRRNAMRLLTVL
Consensus (101)NSTRQS EDLGSQFTEIFIKQ ENVTLLLSLLEEFDFHVRWPGVRLLTSL
151 200
Hsp115 (150)LKQLGPQVQQIILVSPMGVSRLMDLLADSREVIRNDGVLLLQALTRSNGA
Mmp115 (150)LKQLGPPVQQIILVSPMGVSRLMDLLADSREIIRNDGVLLLQALTRSNGA
S.mansoni p115 (151)SINRLKDTQNAVLQCRTGVSKLVDILSDTHEVLRNDVILLLTELTKANAN
Consensus (151)LKQLGP VQQIILVSPMGVSRLMDLLADSREVIRNDGVLLLQALTRSNGA
201 250
Hsp115 (200)IQKIVAFENAFERLLDIISEEGNSDGGIVVEDCLILLQNLLKNNNSNQNF
Mmp115 (200)IQKIVAFENAFERLLDIITEEGNSDGGIVVEDCLILLQNLLKTNNSNQNF
S.mansoni p115 (201)IQKIVAFENAFDWLLKIIYSEGLCDGSVVVEDCLRLLLQLLDGNPSNQVL
Consensus (201)IQKIVAFENAFERLLDIISEEGNSDGGIVVEDCLILLQNLLK NNSNQNF
251 300
Hsp115 (250)FKEGSYIQRMKPWFEVGDENS----GWSAQKVTNLHLMLQLVRVLVSPTN
Mmp115 (250)FKEGSYIQRMKPWFEVGEENS----GWSAQKVTNLHLMLQLVRVLVSPTN
S.mansoni p115 (251)FVEGNFIQRLSLLFDLYSTDSTQPQLWSAQKVVNAEVAMQVVQTLVAPCN
Consensus (251)FKEGSYIQRMKPWFEVGDENS GWSAQKVTNLHLMLQLVRVLVSPTN
301 350
Hsp115 (296)PPGATSSCQKAMFQCGLLQQLCTIILMATGVPADILTETINTVSEVIRGCQ
Mmp115 (296)PPGATSSCQKAMFQCGLLQQLCTIILMATGIPADILTETINTVSEVIRGCQ
S.mansoni p115 (301)KAQLTRNCQNVVYDCGLLEKLCNIVMVSGVPADVLTKSIYALADSIRGCP
Consensus (301)PPGATSSCQKAMFQCGLLQQLCTILMATGVPADILTETINTVSEVIRGCQ
351 400
Hsp115 (346)VNQDYFASVNAPSNPPRPAIVVLLMSMVNERQPFVLRCAVLYCFQCFLYK
Mmp115 (346)VNQDYFASVNAPSNPPRPAIVVLLMSMVNERQPFVLRCAVLYCFQCFLYK
S.mansoni p115 (351)RNQEYLANLVTPSEPPQSAVNVLLVSMLNKHQSVVVRVAALYCFQCYLAS
Consensus (351)VNQDYFASVNAPSNPPRPAIVVLLMSMVNERQPFVLRCAVLYCFQCFLYK
401 450
Hsp115 (396)NQKGQGEIVSTLLPSTIDATGNSVSAGQLLCGGLFSTDSLSNWCAAVALA
Mmp115 (396)NEKGQGEIVATLLPSTIDATGNSVSAGQLLCGGLFSTDSLSNWCAAVALA
S.mansoni p115 (401)NHQAQNAIVMTLLPKPNDVPQLGVSAGQLLCGGLFSSDS-SAWFSSVALL
Consensus (401)N KGQGEIVATLLPSTIDATGNSVSAGQLLCGGLFSTDSLSNWCAAVALA
451 500
Hsp115 (446)HALQENATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQGSKIQTRVGLL
Mmp115 (446)HALQGNATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQGSKIQTRVGLL
S.mansoni p115 (450)HCIHDNVQLKEELLRVHLSPKEGAPPVTLFHQCFMWQQQCNHFQTRMGLL
Consensus (451)HALQDNATQKEQLLRVQLATSIGNPPVSLLQQCTNILSQGSKIQTRVGLL

```
                      501                                                 550
Hsp115          (496)MLLCTWLSNCPIAVTHFLHNSANVP---------------FLTGQIAENL
Mmp115          (496)MLLCTWLSNCPIAVTHFLHNSANVP---------------FLTGQIAENL
S.mansoni p115  (500)QLLCTWFTNCSEAVRCFLNPSSSTGNDRTESVNRSSYLWTLIAEACAVDN
Consensus       (501)MLLCTWLSNCPIAVTHFLHNSANVP               FLTGQIAENL

                       551                                                600
Hsp115          (531)GEEEQLVQGLCALLLGISIYFNDNSLESYMKEKLKQLIEKRIGKENFIEK
Mmp115          (531)GEEEQLVQGLCALLLGISIYFNDNSLENYTKEKLKQLIEKRIGKENFIEK
S.mansoni p115  (550)DENEALVHGLTTLLVCICVMYNPGNVNGFEKSTLYNALEKRIGIDMILER
Consensus       (551)GEEEQLVQGLCALLLGISIYFNDNSLE Y KEKLKQLIEKRIGKENFIEK

                      601                                                 650
Hsp115          (581)LGFISKHELYSRASQKPQPNFPSPEYMIFDHEFTKLVKELEGVITKAIYK
Mmp115          (581)LGFISKHELYSRASQKPQPNFPSPEYMIFDHEFTKLVKELEGVITKAIYK
S.mansoni p115  (600)LSQISKAESFITATKKPQISVQSTDDLIFDYTFTRLFKRLEYEVMHTFQS
Consensus       (601)LGFISKHELYSRASQKPQPNFPSPEYMIFDHEFTKLVKELEGVITKAIYK

                      651                                                 700
Hsp115          (631)SSEEDKKEEEVKKTLEQHDN---IVTHYKNMIREQDLQLEELRQQVSTLK
Mmp115          (631)SSEEDKKEEEVKKTLEQHDN---IVTHYKNMIREQDLQLEELKQQVSTLK
S.mansoni p115  (650)DSSINGVRSCTSFGNNHQNSKPVDPTITTLSQQKYDEKLARQENEIAMLR
Consensus       (651)SSEEDKKEEEVKKTLEQHDN   IVTHYKNMIREQDLQLEELKQQVSTLK

                      701                                                 750
Hsp115          (678)CQNEQLQTAVTQQVSQIQQHKDQYNLLKIQLGKDNQHQGSYSEGAQMNGI
Mmp115          (678)CQNEQLQTAVTQQASQIQQHKDQYNLLKVQLGKDNHHQGSHGDGAQVNGI
S.mansoni p115  (700)NRISVLESELNTCRSTINGNTPTDTKRNNMINEGELQKLTITDNITIQNL
Consensus       (701)CQNEQLQTAVTQQ SQIQQHKDQYNLLKIQLGKDN HQGSHSDGAQINGI

                      751                                                 800
Hsp115          (728)QPEEIGRLREEIEELKRNQELLQSQLTEKDSMIENMKSSQTSGTNEQSSA
Mmp115          (728)QPEEISRLREEIEELKSQQALLQGQLAEKDSLIENLKSSQASGMSEQASA
S.mansoni p115  (750)EQKCSMLEKERDNLRGEHEDLLLLLNDQDVKIMKLCKLVRELGGHIPNDI
Consensus       (751)QPEEI RLREEIEELK NQDLLQ QL EKDSIIENLKSSQ SG  EQASA

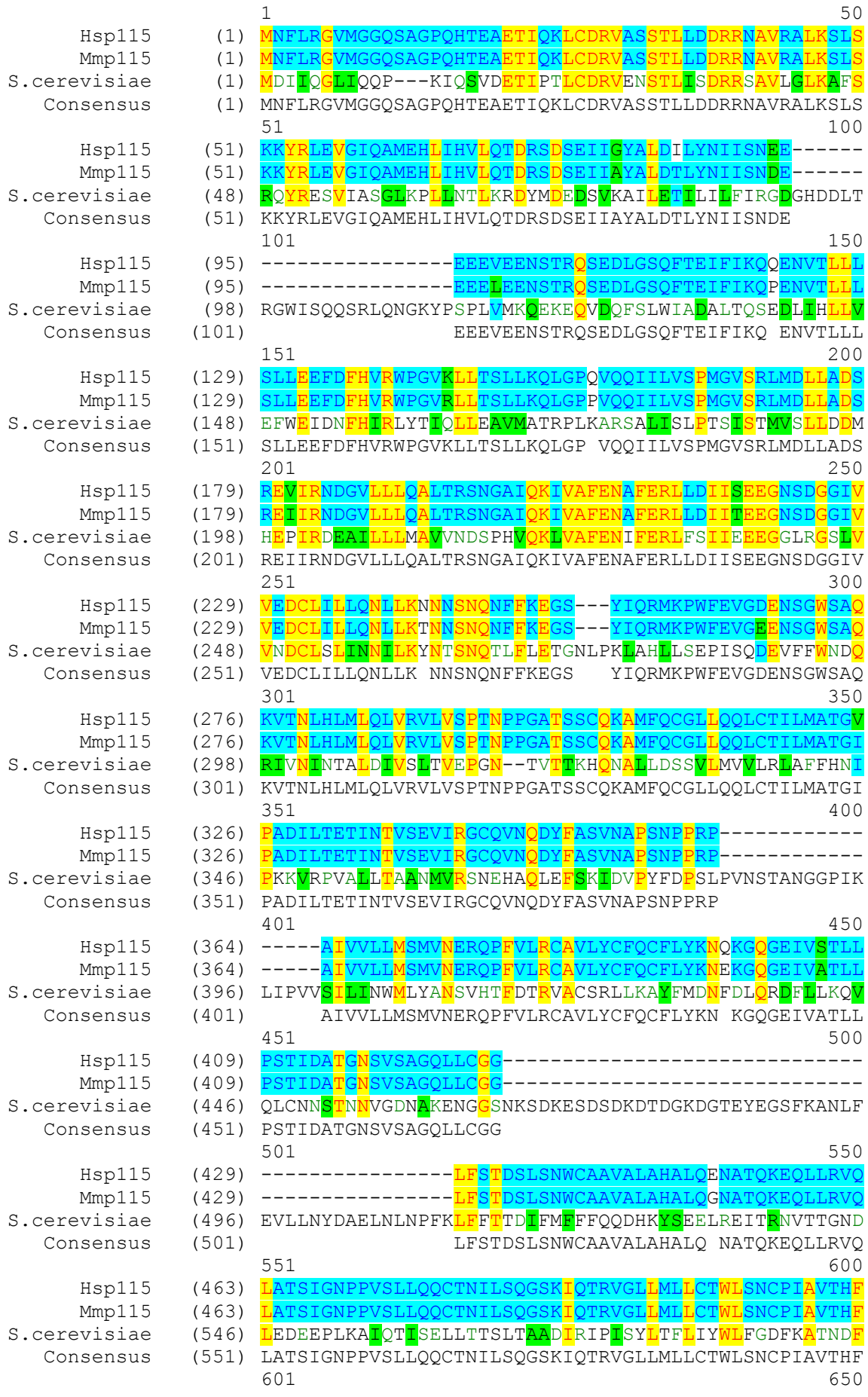
                      801                                                 850
Hsp115          (778)IVSARDSEQVAELKQELATLKSQLNSQSVEITKLQTEKQELLQKTEAFAK
Mmp115          (778)TCPPRDPEQVAELKQELTALKSQLCSQSLEITRLQTENCELLQRAETLAK
S.mansoni p115  (800)GLGNVIDSQVTDNHHNQLSEENTNQSLSTFRTIPSTLPLDTLQCPANFSF
Consensus       (801) L  RD EQVAELKQEL SLKSQLNSQSLEITKLQTE  ELLQK E FAK

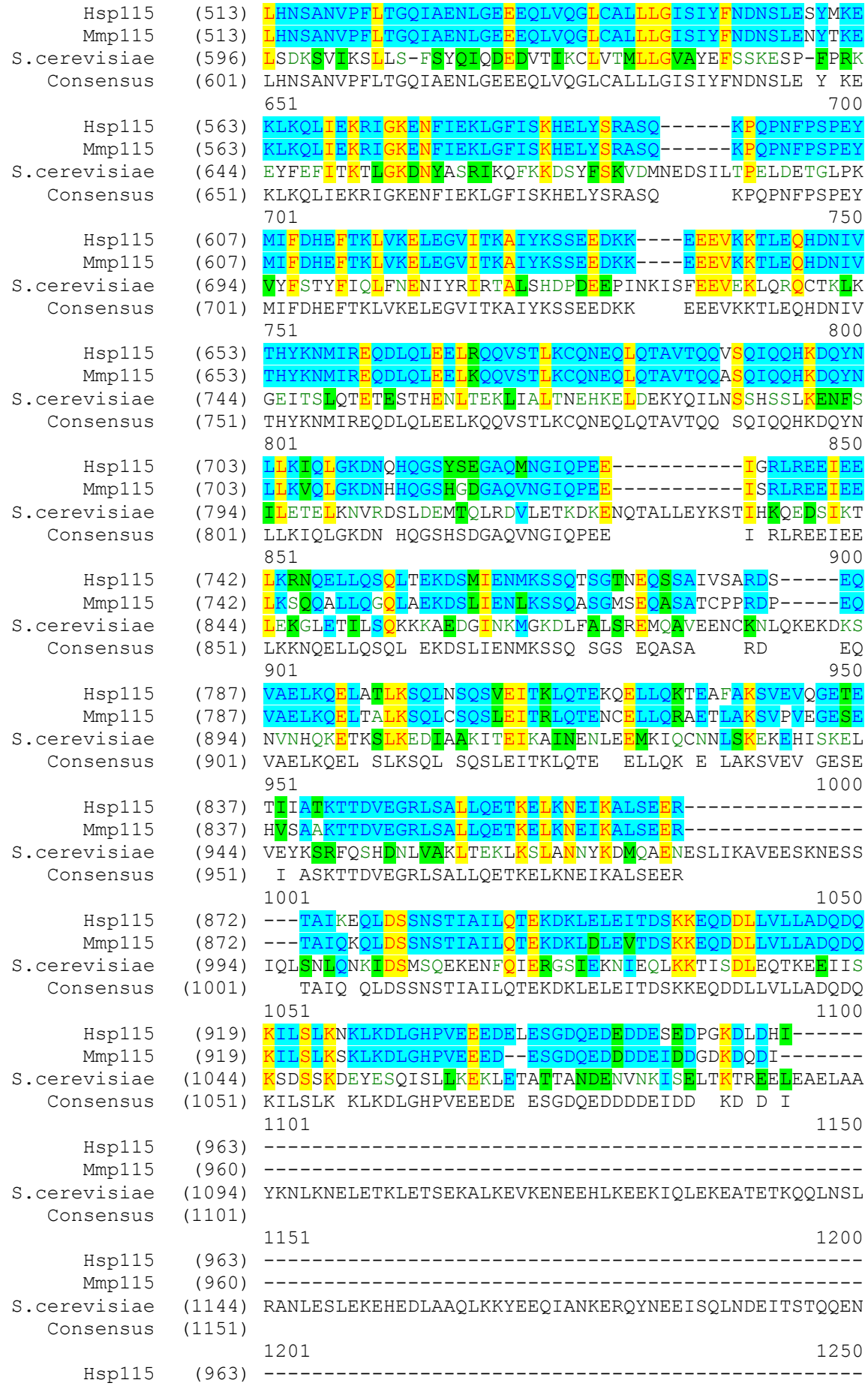
                      851                                                 900
Hsp115          (828)SVEVQGETETIIATKTTDVEGRLSALLQETKELKNEIKALSEERTAIKEQ
Mmp115          (828)SVPVEGESEHVSAAKTTDVEGRLSALLQETKELKNEIKALSEERTAIQKQ
S.mansoni p115  (850)TTSELTLSSNSISTSTPSSYCVYNNNNNDNNNNNNNNNLLHTSPPPLLSH
Consensus       (851)SV V GESE IIATKTTDVEGRLSALLQETKELKNEIKALSEERTAI  Q

                      901                                                 950
Hsp115          (878)LDSSNSTIAILQTEKDKLELEITDSKKEQDDLLVLLADQDQKILSLKNKL
Mmp115          (878)LDSSNSTIAILQTEKDKLDLEVTDSKKEQDDLLVLLADQDQKILSLKSKL
S.mansoni p115  (900)TNSAQNVIMSTTTPTTNQYYYPQGYFNAVTQNYPCSQSSNSVIFYSTNNY
Consensus       (901)LDSSNSTIAILQTEKDKLDLEITDSKKEQDDLLVLLADQDQKILSLKNKL
                      951                                 985
Hsp115          (928)KDLGHPVEEEDELESGDQEDEDDESEDPGKDLDHI
Mmp115          (928)KDLGHPVEEED--ESGDQEDDDDEIDDGDKDQDI-
S.mansoni p115  (950)PIISSNSFNNDHLNSYTNTTIFQ------------
Consensus       (951)KDLGHPVEEED LESGDQEDDDDE DD  KD D
```

Appendix C

Figure C3: Alignment of human (CAA55394) and murine p115 (AAH16069) with *S. mansoni* (XP_002579664) p115 in which 32.4% identity is conserved. Identical amino acids are highlighted in yellow blocks, conservative residues are highlighted in aqua blocks, blocks of similar amino acids are highlighted in green blocks, non-identical amino acids are highlighted in white blocks.


1 50
Hsp115 (1) MNFLRGVMGGQSAGPQHTEAETIQKLCDRVASSTLLDDRRNAVRALKSLS
Mmp115 (1) MNFLRGVMGGQSAGPQHTEAETIQKLCDRVASSTLLDDRRNAVRALKSLS
S.cerevisiae (1) MDIIQGLIQQP---KIQSVDETIPTLCDRVENSTLISDRRSAVLGLKAFS
Consensus (1) MNFLRGVMGGQSAGPQHTEAETIQKLCDRVASSTLLDDRRNAVRALKSLS
51 100
Hsp115 (51) KKYRLEVGIQAMEHLIHVLQTDRSDSEIIGYALDILYNIISNEE------
Mmp115 (51) KKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALDTLYNIISNDE------
S.cerevisiae (48) RQYRESVIASGLKPLLNTLKRDYMDEDSVKAILETILILFIRGDGHDDLT
Consensus (51) KKYRLEVGIQAMEHLIHVLQTDRSDSEIIAYALDTLYNIISNDE
101 150
Hsp115 (95) ----------EEEVEENSTRQSEDLGSQFTEIFIKQQENVTLLL
Mmp115 (95) ----------EEELEENSTRQSEDLGSQFTEIFIKQPENVTLLL
S.cerevisiae (98) RGWISQQSRLQNGKYPSPLVMKQEKEQVDQFSLWIADALTQSEDLIHLLV
Consensus (101) EEEVEENSTRQSEDLGSQFTEIFIKQ ENVTLLL
151 200
Hsp115 (129) SLLEEFDFHVRWPGVKLLTSLLKQLGPQVQQIILVSPMGVSRLMDLLADS
Mmp115 (129) SLLEEFDFHVRWPGVRLLTSLLKQLGPPVQQIILVSPMGVSRLMDLLADS
S.cerevisiae (148) EFWEIDNFHIRLYTIQLLEAVMATRPLKARSALISLPTSISTMVSLLDDM
Consensus (151) SLLEEFDFHVRWPGVKLLTSLLKQLGP VQQIILVSPMGVSRLMDLLADS
201 250
Hsp115 (179) REVIRNDGVLLLQALTRSNGAIQKIVAFENAFERLLDIISEEGNSDGGIV
Mmp115 (179) REIIRNDGVLLLQALTRSNGAIQKIVAFENAFERLLDIITEEGNSDGGIV
S.cerevisiae (198) HEPIRDEAILLLMAVVNDSPHVQKLVAFENIFERLFSIIEEEGGLRGSLV
Consensus (201) REIIRNDGVLLLQALTRSNGAIQKIVAFENAFERLLDIISEEGNSDGGIV
251 300
Hsp115 (229) VEDCLILLQNLLKNNNSNQNFFKEGS---YIQRMKPWFEVGDENSGWSAQ
Mmp115 (229) VEDCLILLQNLLKTNNSNQNFFKEGS---YIQRMKPWFEVGEENSGWSAQ
S.cerevisiae (248) VNDCLSLINNILKYNTSNQTLFLETGNLPKLAHLLSEPISQDEVFFWNDQ
Consensus (251) VEDCLILLQNLLK NNSNQNFFKEGS YIQRMKPWFEVGDENSGWSAQ
301 350
Hsp115 (276) KVTNLHLMLQLVRVLVSPTNPPGATSSCQKAMFQCGLLQQLCTILMATGV
Mmp115 (276) KVTNLHLMLQLVRVLVSPTNPPGATSSCQKAMFQCGLLQQLCTILMATGI
S.cerevisiae (298) RIVNINTALDIVSLTVEPGN--TVTTKHQNALLDSSVLMVVLRLAFFHNI
Consensus (301) KVTNLHLMLQLVRVLVSPTNPPGATSSCQKAMFQCGLLQQLCTILMATGI
351 400
Hsp115 (326) PADILTETINTVSEVIRGCQVNQDYFASVNAPSNPPRP----------
Mmp115 (326) PADILTETINTVSEVIRGCQVNQDYFASVNAPSNPPRP----------
S.cerevisiae (346) PKKVRPVALLTAANMVRSNEHAQLEFSKIDVPYFDPSLPVNSTANGGPIK
Consensus (351) PADILTETINTVSEVIRGCQVNQDYFASVNAPSNPPRP
401 450
Hsp115 (364) -----AIVVLLMSMVNERQPFVLRCAVLYCFQCFLYKNQKGQGEIVSTLL
Mmp115 (364) -----AIVVLLMSMVNERQPFVLRCAVLYCFQCFLYKNEKGQGEIVATLL
S.cerevisiae (396) LIPVVSILINWMLYANSVHTFDTRVACSRLLKAYFMDNFDLQRDFLLKQV
Consensus (401) AIVVLLMSMVNERQPFVLRCAVLYCFQCFLYKN KGQGEIVATLL
451 500
Hsp115 (409) PSTIDATGNSVSAGQLLCGG----------
Mmp115 (409) PSTIDATGNSVSAGQLLCGG----------
S.cerevisiae (446) QLCNNSTNNVGDNAKENGGSNKSDKESDSDKDTDGKDGTEYEGSFKANLF
Consensus (451) PSTIDATGNSVSAGQLLCGG
501 550
Hsp115 (429) ----------LFSTDSLSNWCAAVALAHALQENATQKEQLLRVQ
Mmp115 (429) ----------LFSTDSLSNWCAAVALAHALQGNATQKEQLLRVQ
S.cerevisiae (496) EVLLNYDAELNLNPFKLFFTTDIFMFFFQQDHKYSEELREITRNVTTGND
Consensus (501) LFSTDSLSNWCAAVALAHALQ NATQKEQLLRVQ
551 600
Hsp115 (463) LATSIGNPPVSLLQQCTNILSQGSKIQTRVGLLMLLCTWLSNCPIAVTHF
Mmp115 (463) LATSIGNPPVSLLQQCTNILSQGSKIQTRVGLLMLLCTWLSNCPIAVTHF
S.cerevisiae (546) LEDEEPLKAIQTISELLTTSLTAADIRIPISYLTFLIYWLFGDFKATNDF
Consensus (551) LATSIGNPPVSLLQQCTNILSQGSKIQTRVGLLMLLCTWLSNCPIAVTHF
601 650

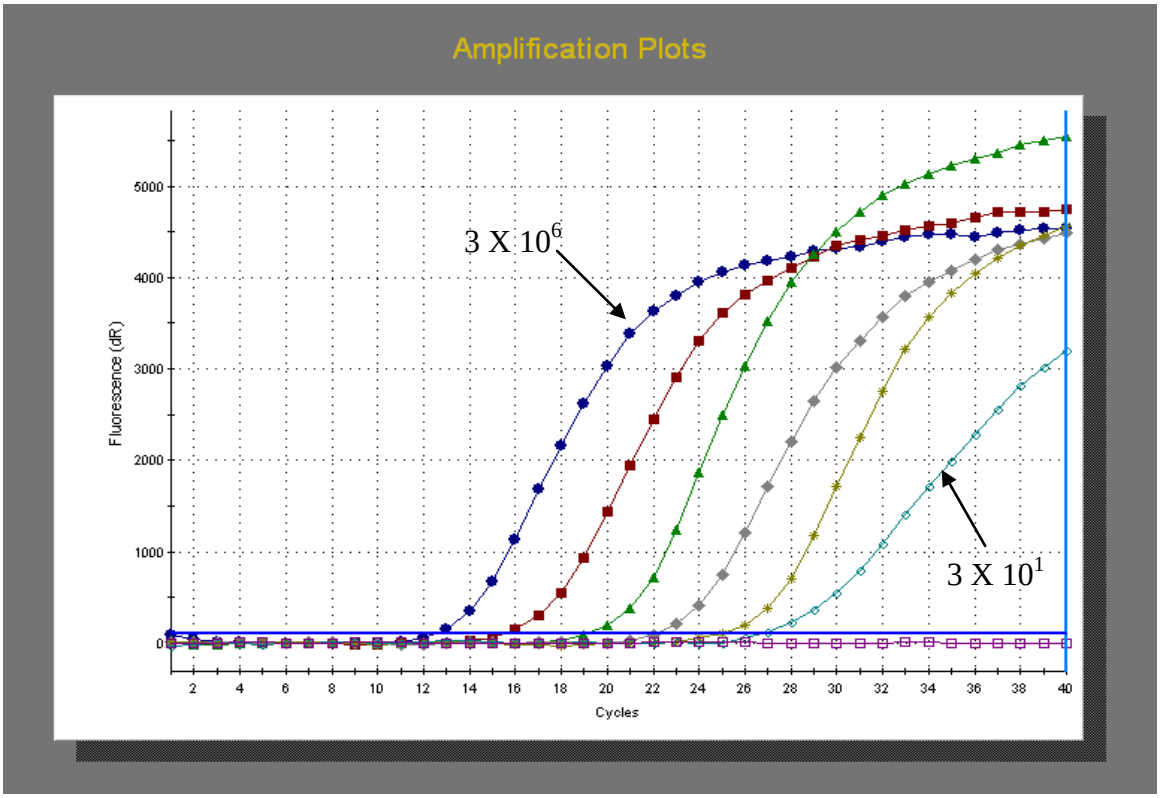

Hsp115 (513) LHNSANVPFLTGQIAENLGEEEQLVQGLCALLLGISIYFNDNSLESYMKE
Mmp115 (513) LHNSANVPFLTGQIAENLGEEEQLVQGLCALLLGISIYFNDNSLENYTKE
S.cerevisiae (596) LSDKSVIKSLLS-FSYQIQDEDVTIKCLVTMLLGVAYEFSSKESP-FPRK
Consensus (601) LHNSANVPFLTGQIAENLGEEEQLVQGLCALLLGISIYFNDNSLE Y KE
651 700
Hsp115 (563) KLKQLIEKRIGKENFIEKLGFISKHELYSRASQ------KPQPNFPSPEY
Mmp115 (563) KLKQLIEKRIGKENFIEKLGFISKHELYSRASQ------KPQPNFPSPEY
S.cerevisiae (644) EYFEFITKTLGKDNYASRIKQFKKDSYFSKVDMNEDSILTPELDETGLPK
Consensus (651) KLKQLIEKRIGKENFIEKLGFISKHELYSRASQ KPQPNFPSPEY
701 750
Hsp115 (607) MIFDHEFTKLVKELEGVITKAIYKSSEEDKK----EEEVKKTLEQHDNIV
Mmp115 (607) MIFDHEFTKLVKELEGVITKAIYKSSEEDKK----EEEVKKTLEQHDNIV
S.cerevisiae (694) VYFSTYFIQLFNENIYRIRTALSHDPDEEPINKISFEEVEKLQRQCTKLK
Consensus (701) MIFDHEFTKLVKELEGVITKAIYKSSEEDKK EEEVKKTLEQHDNIV
751 800
Hsp115 (653) THYKNMIREQDLQLEELRQQVSTLKCQNEQLQTAVTQQVSQIQQHKDQYN
Mmp115 (653) THYKNMIREQDLQLEELKQQVSTLKCQNEQLQTAVTQQASQIQQHKDQYN
S.cerevisiae (744) GEITSLQTETESTHENLTEKLIALTNEHKELDEKYQILNSSHSSLKENFS
Consensus (751) THYKNMIREQDLQLEELKQQVSTLKCQNEQLQTAVTQQ SQIQQHKDQYN
801 850
Hsp115 (703) LLKIQLGKDNQHQGSYSEGAQMNGIQPEE-----------IGRLREEIEE
Mmp115 (703) LLKVQLGKDNHHQGSHGDGAQVNGIQPEE-----------ISRLREEIEE
S.cerevisiae (794) ILETELKNVRDSLDEMTQLRDVLETKDKENQTALLEYKSTIHKQEDSIKT
Consensus (801) LLKIQLGKDN HQGSHSDGAQVNGIQPEE I RLREEIEE
851 900
Hsp115 (742) LKRNQELLQSQLTEKDSMIENMKSSQTSGTNEQSSAIVSARDS-----EQ
Mmp115 (742) LKSQQALLQGQLAEKDSLIENLKSSQASGMSEQASATCPPRDP-----EQ
S.cerevisiae (844) LEKGLETILSQKKKAEDGINKMGKDLFALSREMQAVEENCKNLQKEKDKS
Consensus (851) LKKNQELLQSQL EKDSLIENMKSSQ SGS EQASA RD EQ
901 950
Hsp115 (787) VAELKQELATLKSQLNSQSVEITKLQTEKQELLQKTEAFAKSVEVQGETE
Mmp115 (787) VAELKQELTALKSQLCSQSLEITRLQTENCELLQRAETLAKSVPVEGESE
S.cerevisiae (894) NVNHQKETKSLKEDIAAKITEIKAINENLEEMKIQCNNLSKEKEHISKEL
Consensus (901) VAELKQEL SLKSQL SQSLEITKLQTE ELLQK E LAKSVEV GESE
951 1000
Hsp115 (837) TIIATKTTDVEGRLSALLQETKELKNEIKALSEER----------
Mmp115 (837) HVSAAKTTDVEGRLSALLQETKELKNEIKALSEER----------
S.cerevisiae (944) VEYKSRFQSHDNLVAKLTEKLKSLANNYKDMQAENESLIKAVEESKNESS
Consensus (951) I ASKTTDVEGRLSALLQETKELKNEIKALSEER
1001 1050
Hsp115 (872) ---TAIKEQLDSSNSTIAILQTEKDKLELEITDSKKEQDDLLVLLADQDQ
Mmp115 (872) ---TAIQKQLDSSNSTIAILQTEKDKLDLEVTDSKKEQDDLLVLLADQDQ
S.cerevisiae (994) IQLSNLQNKIDSMSQEKENFQIERGSIEKNIEQLKKTISDLEQTKEEIIS
Consensus (1001) TAIQ QLDSSNSTIAILQTEKDKLELEITDSKKEQDDLLVLLADQDQ
1051 1100
Hsp115 (919) KILSLKNKLKDLGHPVEEEDELESGDQEDEDDESEDPGKDLDHI------
Mmp115 (919) KILSLKSKLKDLGHPVEEED--ESGDQEDDDDEIDDGDKDQDI-------
S.cerevisiae (1044) KSDSSKDEYESQISLLKEKLETATTANDENVNKISELTKTREELEAELAA
Consensus (1051) KILSLK KLKDLGHPVEEEDE ESGDQEDDDDEIDD KD D I
1101 1150
Hsp115 (963) ----------
Mmp115 (960) ----------
S.cerevisiae (1094) YKNLKNELETKLETSEKALKEVKENEEHLKEEKIQLEKEATETKQQLNSL
Consensus (1101)
1151 1200
Hsp115 (963) ----------
Mmp115 (960) ----------
S.cerevisiae (1144) RANLESLEKEHEDLAAQLKKYEEQIANKERQYNEEISQLNDEITSTQQEN
Consensus (1151)
1201 1250
Hsp115 (963) ----------

```
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1194) ESIKKKNDELEGEVKAMKSTSEEQSNLKKSEIDALNLQIKELKKKNETNE
   Consensus  (1201)
                       1251                                        1300
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1244) ASLLESIKSVESETVKIKELQDECNFKEKEVSELEDKLKASEDKNSKYLE
   Consensus  (1251)
                       1301                                        1350
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1294) LQKESEKIKEELDAKTTELKIQLEKITNLSKAKEKSESELSRLKKTSSEE
   Consensus  (1301)
                       1351                                        1400
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1344) RKNAEEQLEKLKNEIQIKNQAFEKERKLLNEGSSTITQEYSEKINTLEDE
   Consensus  (1351)
                       1401                                        1450
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1394) LIRLQNENELKAKEIDNTRSELEKVSLSNDELLEEKQNTIKSLQDEILSY
   Consensus  (1401)
                       1451                                        1500
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1444) KDKITRNDEKLLSIERDNKRDLESLKEQLRAAQESKAKVEEGLKKLEEES
   Consensus  (1451)
                       1501                                        1550
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1494) SKEKAELEKSKEMMKKLESTIESNETELKSSMETIRKSDEKLEQSKKSAE
   Consensus  (1501)
                       1551                                        1600
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1544) EDIKNLQHEKSDLISRINESEKDIEELKSKLRIEAKSGSELETVKQELNN
   Consensus  (1551)
                       1601                                        1650
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1594) AQEKIRINAEENTVLKSKLEDIERELKDKQAEIKSNQEEKELLTSRLKEL
   Consensus  (1601)
                       1651                                        1700
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1644) EQELDSTQQKAQKSEEERRAEVRKFQVEKSQLDEKAMLLETKYNDLVNKE
   Consensus  (1651)
                       1701                                        1750
      Hsp115   (963) --------------------------------------------------
      Mmp115   (960) --------------------------------------------------
S.cerevisiae  (1694) QAWKRDEDTVKKTTDSQRQEIEKLAKELDNLKAENSKLKEANEDRSEIDD
   Consensus  (1701)
                       1751                                     1797
      Hsp115   (963) ---------------------------------------------
      Mmp115   (960) ---------------------------------------------
S.cerevisiae  (1744) LMLLVTDLDEKNAKYRSKLKDLGVEISSDEEDDEEDDEEDEEEGQVA
   Consensus  (1751)
```

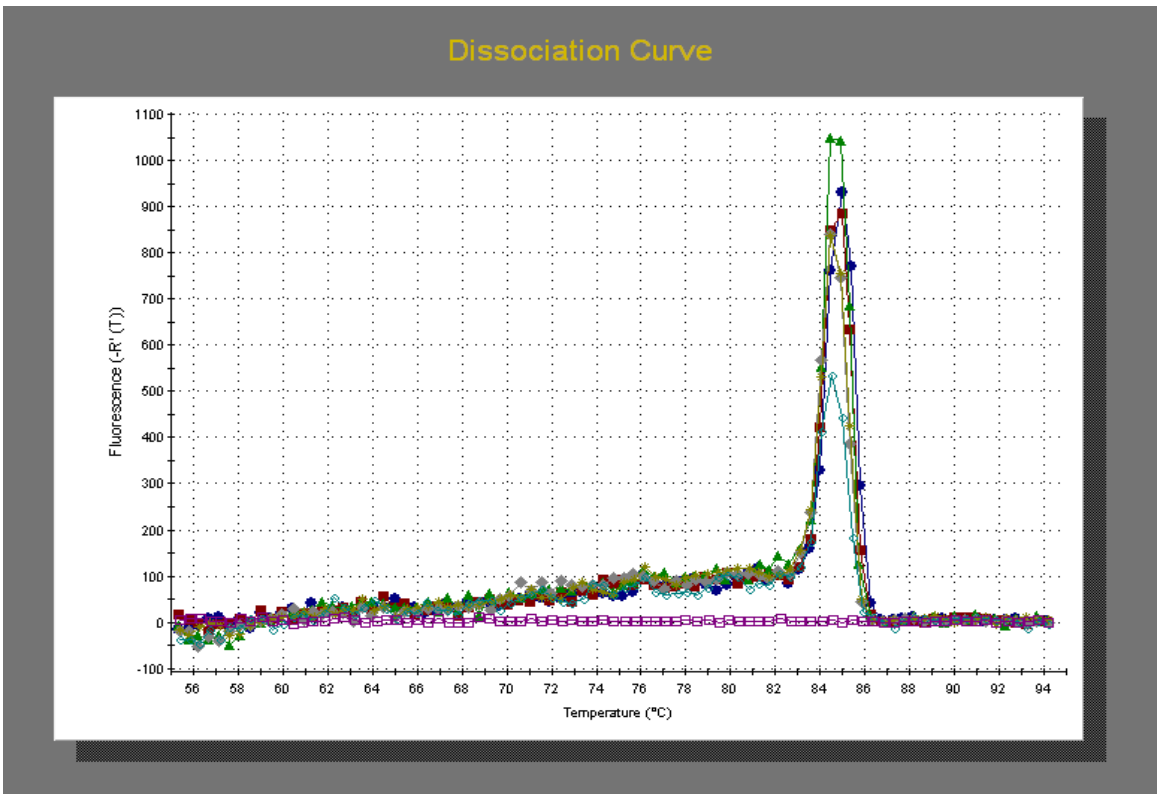
Appendix C

Figure C4: Alignment of human (CAA55394) and murine p115 (AAH16069) with *S. cerevisiae* (CAA98621) p115 in which 10.8% identity is conserved. Identical amino acids are highlighted in yellow blocks, conservative residues are highlighted in aqua blocks, blocks of similar amino acids are highlighted in green blocks, non-identical amino acids are highlighted in white blocks.

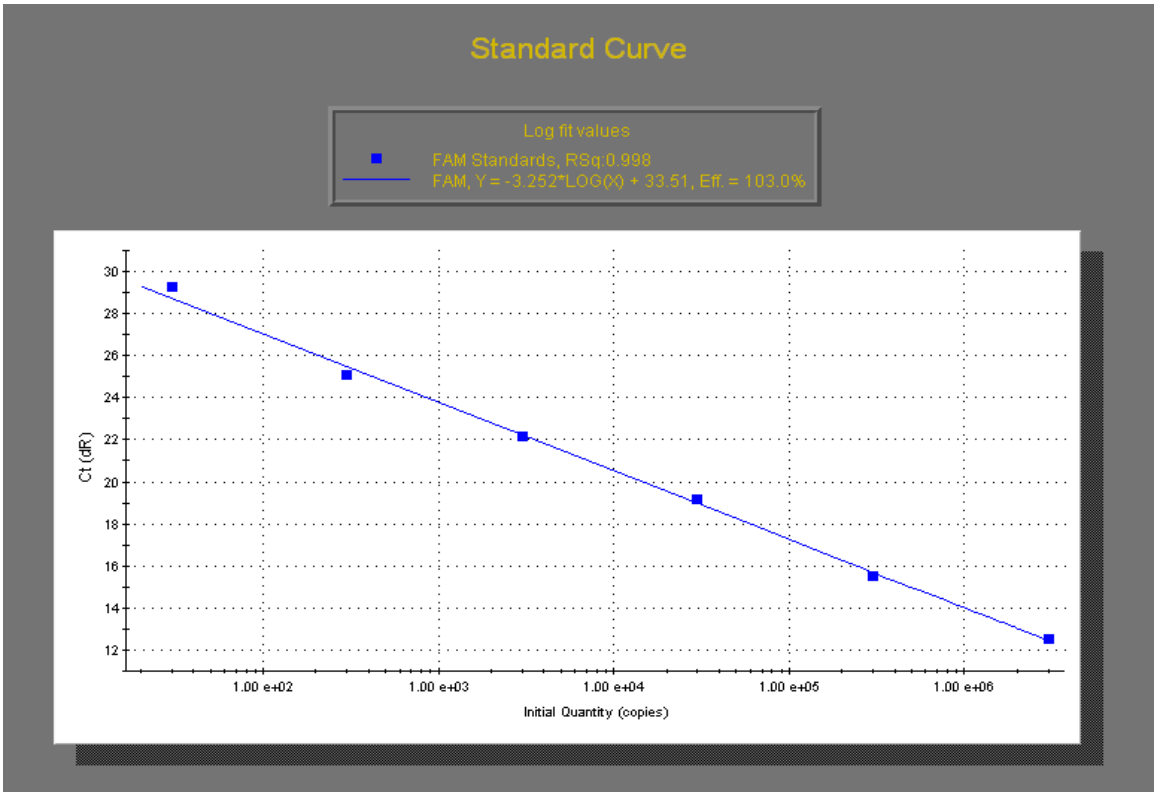
A


Amplification Plots
Fluorescence (dR)
Cycles
3 X 10^6
3 X 10^1

B


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

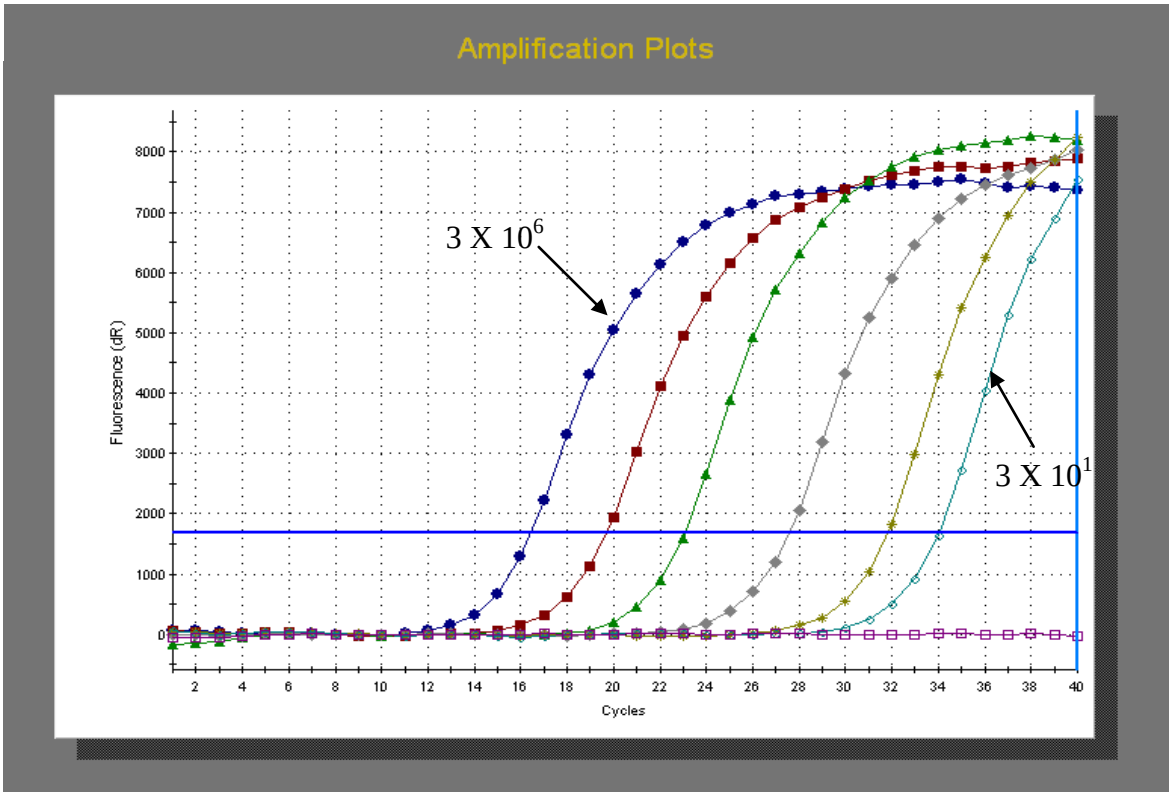
C



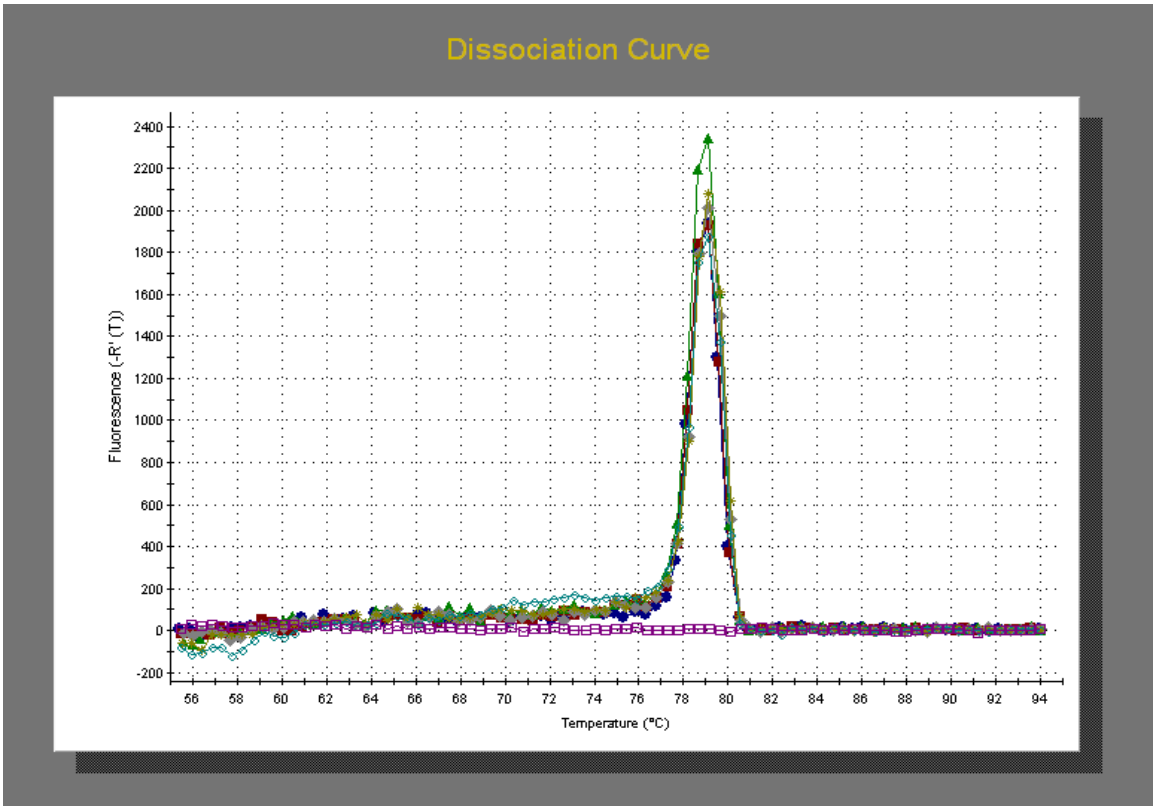
Appendix D

Figure D1: A portion of TBP was amplified by conventional PCR and used to make a standard curve with standards ranging from $3x10^6$ to $3x10^1$ copies. Standards were tested resulting in the generation of an amplification curve (Figure D1(A)) which indicates the order in which DNA is amplified to a detectable level. A melting curve is also generated which represents the dissociation characteristics of dsDNA during the process of denaturation (Figure D1(B)) whereby the presence of one peak represents the amplification of a single, specific PCR product. Figure D1(C) shows a TBP standard curve in which $R^2 = 0.998$.

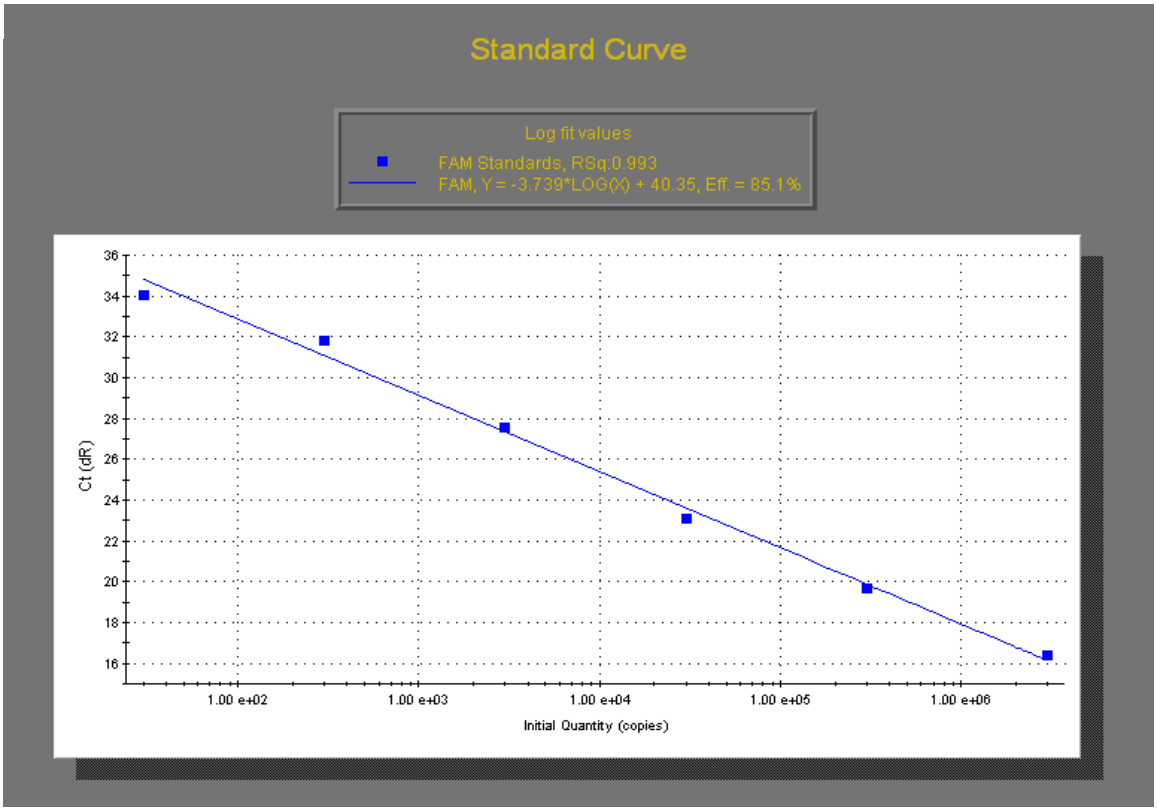
A


Amplification Plots
Fluorescence (dR)
Cycles
3 X 10^6
3 X 10^1

B


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

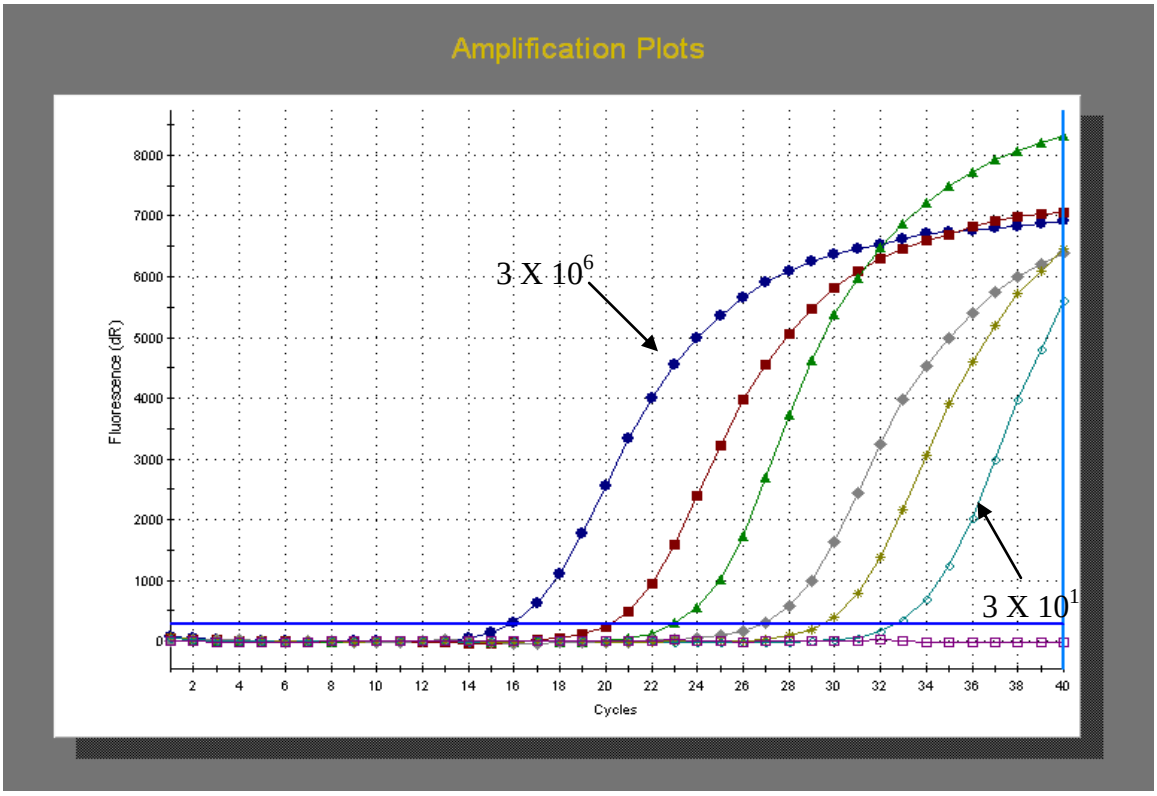
C



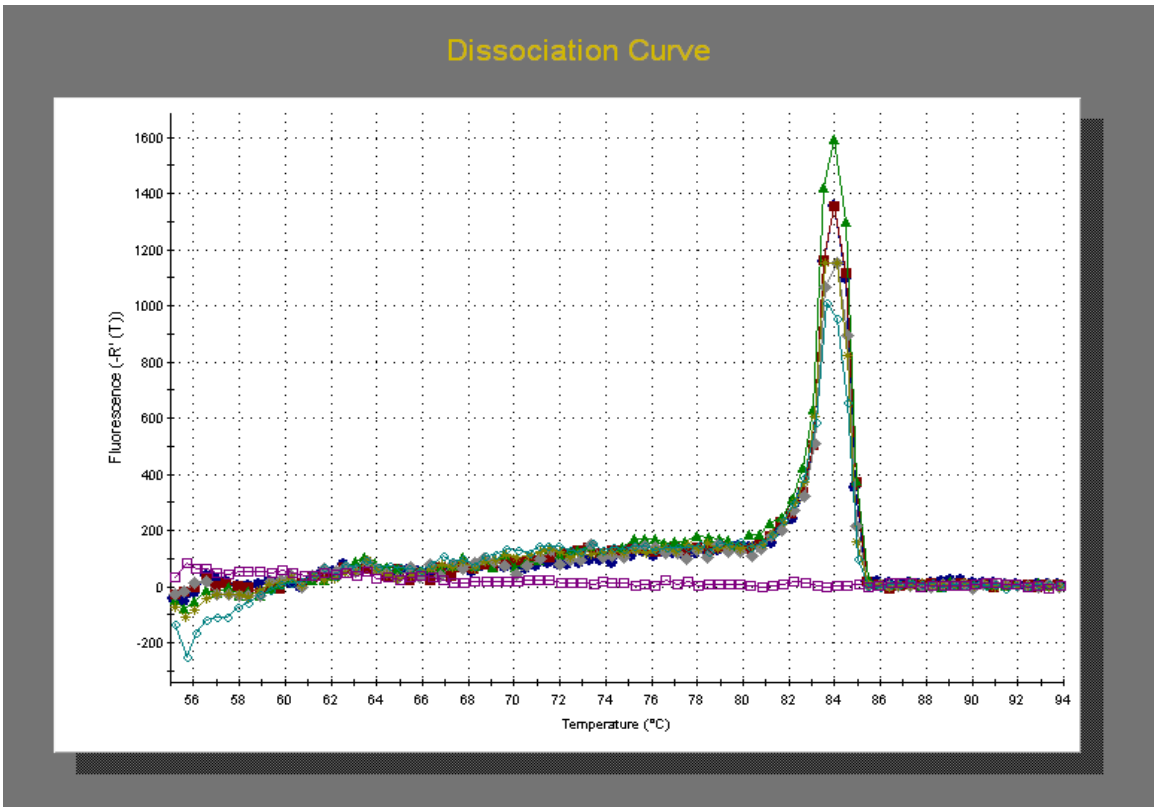
Appendix D

Figure D2: A portion of TLR-4 was amplified by conventional PCR and used to make a standard curve with standards ranging from $3x10^6$ to $3x10^1$ copies. Standards were tested resulting in the generation of an amplification curve (Figure D2(A)) which indicates the order in which DNA is amplified to a detectable level. A melting curve is also generated which represents the dissociation characteristics of dsDNA during the process of denaturation (Figure D2(B)) whereby the presence of one peak represents the amplification of a single, specific PCR product. Figure D2(C) shows a TLR-4 standard curve in which $R^2 = 0.993$.

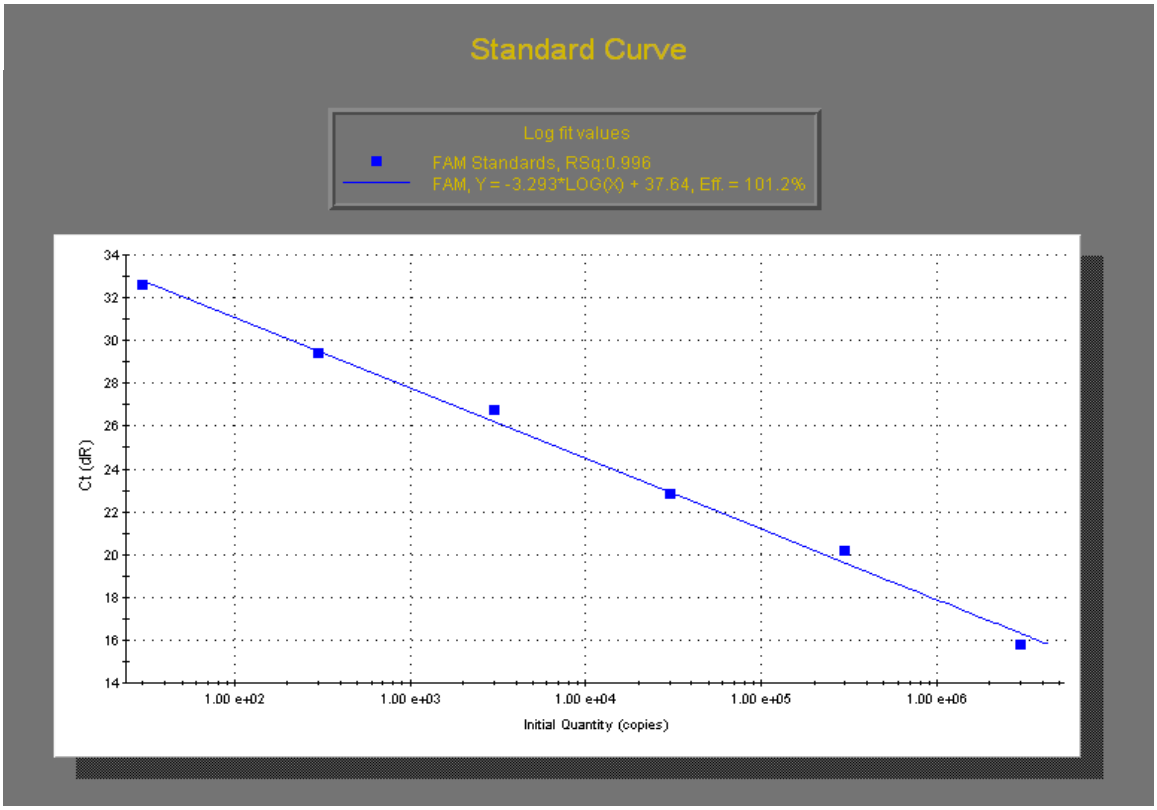
A


Amplification Plots
Fluorescence (dR)
Cycles
3 X 10^6
3 X 10^1

B


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

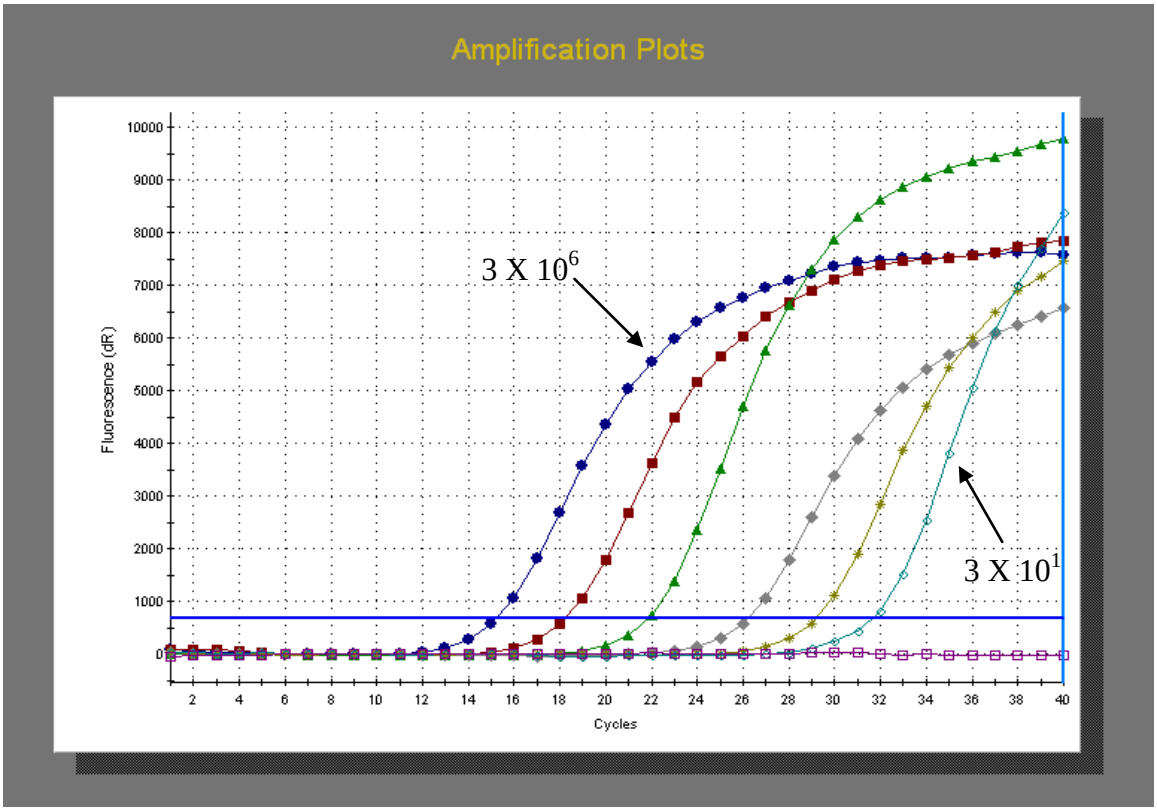
C



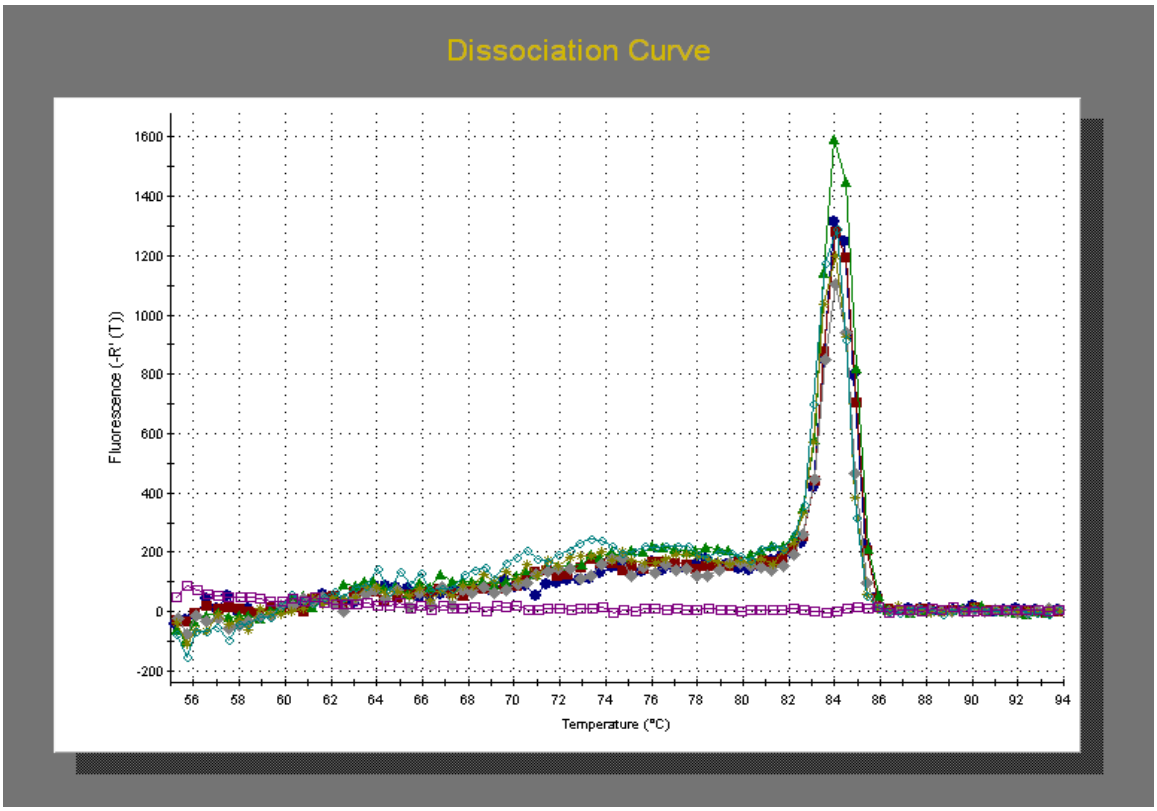
Appendix D

Figure D3: A portion of CD74 was amplified by conventional PCR and used to make a standard curve with standards ranging from $3x10^6$ to $3x10^1$ copies. Standards were tested resulting in the generation of an amplification curve (Figure D3(A)) which indicates the order in which DNA is amplified to a detectable level. A melting curve is also generated which represents the dissociation characteristics of dsDNA during the process of denaturation (Figure D3(B)) whereby the presence of one peak represents the amplification of a single, specific PCR product. Figure D3(C) shows a CD74 standard curve in which $R^2 = 0.996$.

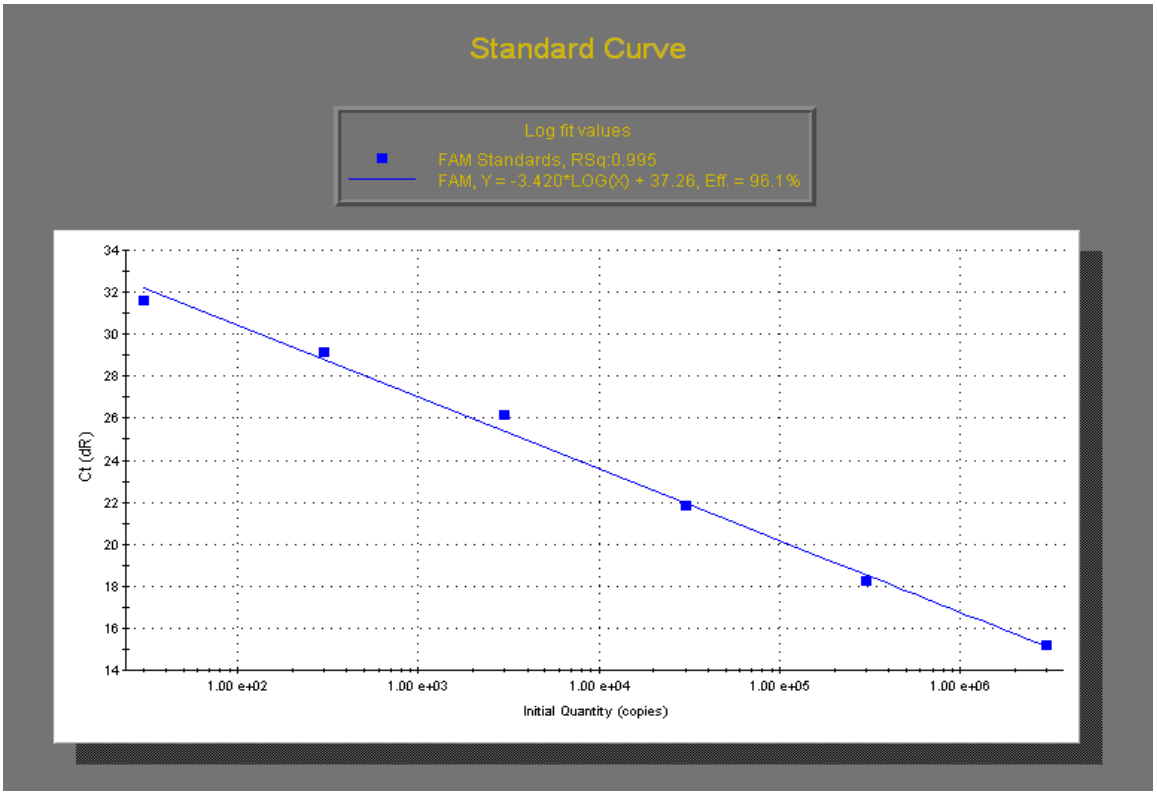
A


Amplification Plots
Fluorescence (dR)
Cycles
3 X 10^6
3 X 10^1

B

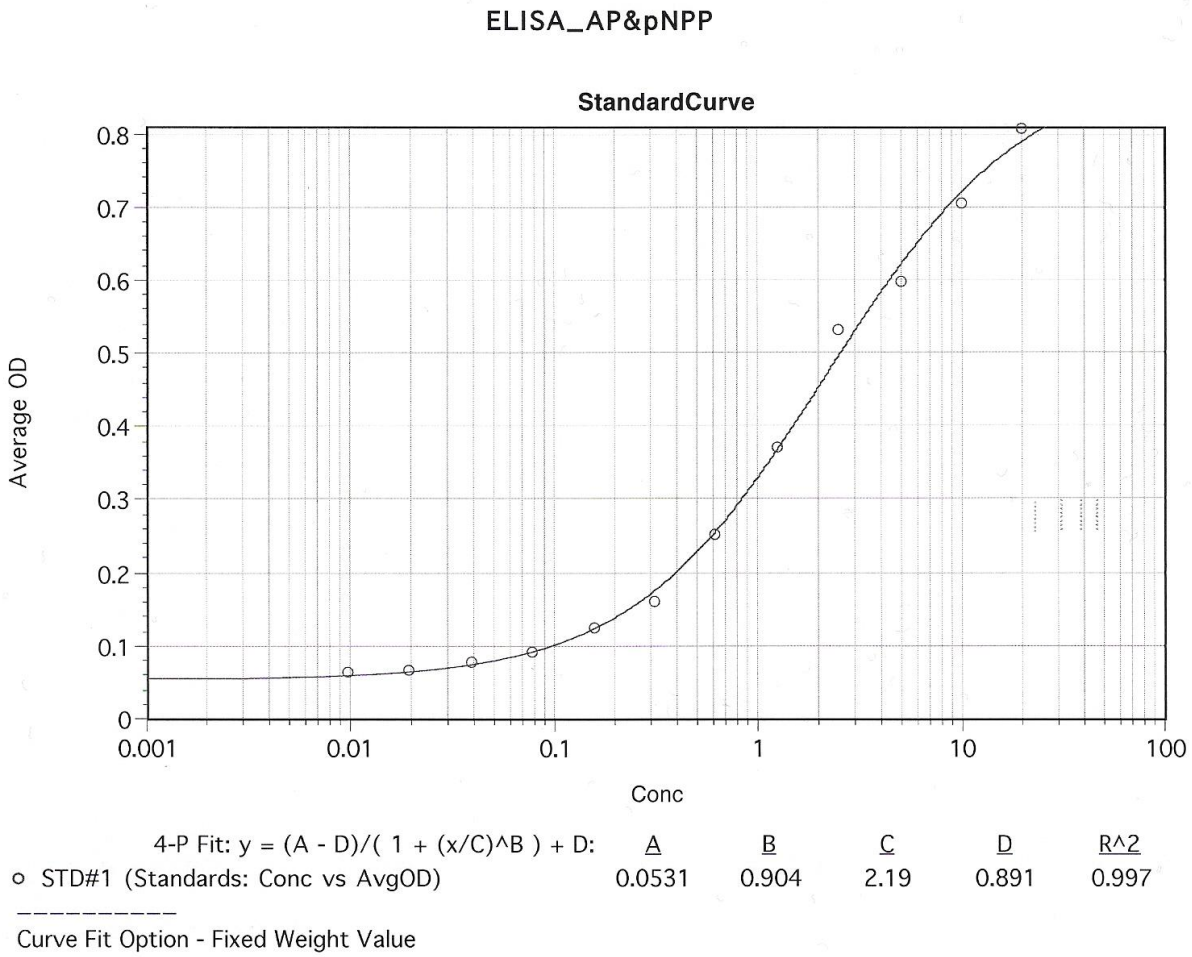

Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

C



Appendix D

Figure D4: A portion of CD44 was amplified by conventional PCR and used to make a standard curve with standards ranging from $3x10^6$ to $3x10^1$ copies. Standards were tested resulting in the generation of an amplification curve (Figure D4(A)) which indicates the order in which DNA is amplified to a detectable level. A melting curve is also generated which represents the dissociation characteristics of dsDNA during the process of denaturation (Figure D4(B)) whereby the presence of one peak represents the amplification of a single, specific PCR product. Figure D4(C) shows a CD44 standard curve in which $R^2 = 0.995$.



Appendix E

Figure E: IL-8 standard curve ranging from 20ng/ml to0.02ng/ml. $R^2 = 0.997$

Crystal symmetry		
Space group	H3(2)	
Unit cell dimensions (Å)	a,b=58.73; c= 183.97	
Vm	2.33	
% solvent	47.3	
	Overall	**OuterShell**
Low resolution limit	49.03	1.92
High resolution limit	1.82	1.82
Wavelength		
Rmerge	0.083	0.395
Total observations	117082	17090
Unique observations	11318	1615
I/σ(I)	22.4	5.8
Completeness (%)	100.0	100.0
Multiplicity	10.3	10.6
Refinement		
R (%)	19.3	
Rfree (%)	23.1	
Bond length r.m.s.d. from ideality (Å)	0.008	
Bond angle r.m.s.d. from ideality (deg)	1.066	
Rmsd chiral	0.080	
Average B factor ($Å^2$)	19.34	
Ramachandran plot core (%)	91.1	
Allowed (%)	8.9	
Outlier (%)	0	

Appendix F

Table F: Parameters for crystallisation studies.

pH	Xml 0.2M Na_2HPO_4	Yml 0.2M NaH_2PO_4
5.8	4.0	46.0
6.0	6.15	43.85
6.2	9.25	40.75
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.75	4.25
8.0	47.35	2.65

Appendix G

Table G: Table used for the preparation of sodium phosphate buffers in tautomerase assays.